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Active site studies and mechanism of action of Leuconostoc mesenteroides B-512FM dextransucrase

Daotian Fu
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Active site studies and mechanism of action of *Leuconostoc mesenteroides* B-512FM dextransucrase

by

Daotian Fu

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ABBREVIATIONS USED

C-1	carbon 1 on the glucosyl residue
C-2	carbon 2 on the glucosyl residue
C-3	carbon 3 on the glucosyl residue
C-4	carbon 4 on the glucosyl residue
C-6	carbon 6 on the glucosyl residue
^{13}C	carbon 13
^{14}C	carbon 14
D_2O	deuterium oxide
DEAE	diethylaminoethyl
DEP	diethyl pyrocarbonate
DP	degree of polymerization
Fru	fructose
Glc	glucose
G2	maltose
G3	maltotriose
G4	maltotetraose
G5	maltopentaose
G6	maltohexaose
G7	maltoheptaose
G8	maltooctaose
IU	international unit of enzymatic activity
kDa	kilodaltons
Ki	inhibition constant
Km	Michaelis binding constant
Leu	leucrose
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
PPA	porcine pancreatic α-amylase
TLC	thin layer chromatography

GENERAL INTRODUCTION

One of the challenges of biochemistry is to develop a fundamental understanding of the relationship between structure and function of enzymes in order to be able to predict the catalytic properties of new enzymes. While genetic engineering and X-ray diffraction can be used to study these relationships, the majority of the information has come from conventional biochemical analyses of enzymes. The normal steps in this process involve establishing kinetic parameters, obtaining structural information, probing the active site with substrate analogues, and modeling the catalytic behavior. In the case of dextransucrase, the crystalline form has not yet been obtained, and little structural information has been available. Studies of its active site and the mechanism of action have, therefore, become the first step.

The dextransucrase from Leuconostoc mesenteroides NRRL B-512FM polymerizes the glucosyl moiety of sucrose to produce dextran, a D-glucan with 95% $\alpha(1\rightarrow6)$ linkages and 5% $\alpha(1\rightarrow3)$ branches[1]. L. mesenteroides B-512FM secretes only one glucansucrase, and compared to most glucansucrase producing bacteria, it secretes only small amounts of other enzymes that act on sucrose or dextran. This makes the B-512FM dextransucrase an unusually good candidate for purification and kinetic studies. Further, research on dextransucrase is of significant medical and commercial importance since it plays a major role in dental caries formation[2,3], and its dextran is used commercially as a blood plasma extender and to make dextran sulfate and Sephadex[4].

The mechanism of action of dextransucrase has attracted much attention during the past half decade. Dextran synthesis was initially assumed to occur at the nonreducing

end[5-7]. In 1968, Ebert and Schenk challenged the mechanism by proposing a reducing end synthesis model[8]. Since then experimental evidence has been presented to support the reducing end synthesis model[9,10], and a mechanism has been proposed for this reaction by Robyt, Walseth and Kimble[9-11]. In this model, it has been proposed that two nucleophiles in the active site attack bound sucrose molecules to give ß-glucosyl-enzyme intermediates covalently linked through C-1. At the same time, two proton donors each donate a proton to the glycosidic oxygen of each sucrose to release the two fructose molecules. During the dextran synthesis, the C-6 hydroxyl on one of the covalently linked glucosyl residues acts as a nucleophile to attack the C-1 of the other glucosyl unit, thereby forming an $\alpha(1\rightarrow6)$ glucosidic bond. The unprotonated proton donor abstracts a proton from the C-6 hydroxyl in this process to restore its original protonated form. This process of forming α-(1→6) linkage releases one of the nucleophilic group, which then can attack another sucrose molecule forming a new glucosyl-enzyme intermediate. The C-6 hydroxyl of this new glucosyl unit then attacks the C-1 of the isomaltosyl unit, forming an isomaltotriosyl-enzyme complex. This process continues with the two catalytic groups alternately forming covalent complexes with glucose and dextran. Chain elongation will be terminated when the dextran is released from the enzyme. This will occur when either fructose or glucose reaches sufficient concentration that their C-5 or C-6 hydroxyl groups, respectively, make a nucleophilic attack on the C-1 of the reducing end residue of dextran.

If other carbohydrate molecules are present and come to the active site, one of their hydroxyl groups will act as a nucleophile to displace the glucosyl or dextranosyl group from the active site to form products. These carbohydrate

molecules are called acceptors, and consequently the products thus formed are called acceptor products. The acceptors range in size from monosaccharides to polysaccharides[12-16]. When the acceptor is a monosaccharide, or a small oligosaccharide, one usually obtains a homologous series of acceptor products containing $\alpha(1\rightarrow6)$ linkages. In contrast, when dextran is an acceptor, one obtains glucosyl or glucanosyl transfer along the polymer backbone[15,16], giving branching rather than elongation.

The sucrose binding site of dextransucrase has been probed with various sucrose analogues[17,18]. The nature of the proton donors in the active site, however, is not yet known. Further, there is little information available about the acceptor binding site. A better understanding of those questions will lead to a clearer picture of the enzyme's two different mechanisms of dextran polymerization and acceptor reactions, and in the future development of anti-dental caries agents. It is towards these goals that this research has been performed.

Explanation of Dissertation Format

This dissertation is presented in four sections, each of which has been published or submitted for publication as an individual paper in a scientific journal. References for each section are listed at its end, except for GENERAL INTRODUCTION, LITERATURE REVIEW, AND GENERAL CONCLUSION, for which references are listed in ADDITIONAL REFERENCES, near the end of the dissertation.

The work described in this dissertation was performed entirely by myself, under the guidance of Dr. John F. Robyt, with the exception of SECTION II, where Dr. Morey E. Slodki from the Northern Regional Research Center, U. S. Department of Agriculture (Peoria, IL) kindly performed methylation

analysis of the acceptor products. All of the papers were prepared by myself, with the assistance of Dr. John F. Robyt in editing and revising the manuscript.

LITERATURE REVIEW

Purification of Dextransucrase

Since the discovery of dextransucrase in 1941[5], it has gained importance because dextran and modified dextran have found many industrial and medical uses. In the early study of L. mesenteroides B-512F dextransucrase, relatively crude enzymes were prepared by alcohol precipitation of the culture supernatant solution[19-21]. Since the dextransucrase from this organism is an inducible enzyme that requires sucrose in the culture medium, and results in large amounts of dextran being formed in the culture supernatant, the enzyme purified by alcohol precipitation inevitably carried a significant amount of dextran with the enzyme. Braswell et al.[21] purified the alcohol precipitated enzyme by adsorption onto calcium phosphate gels. The eluted enzyme was claimed to be free of dextran as judged by a serological test, which has not been well characterized since it is known that B-512F dextran is a very weak antigen and gives poor serological reactions[22]. The non-dextran carbohydrate was not assayed.

It was only after Ebert and Schenk[23] purified dextransucrase that the purification of this enzyme has been extensively studied, and relatively high specific activities were obtained. Their scheme, however, was tedious and resulted in low yields and insoluble enzyme precipitates.

In 1979, Robyt and Walseth[24] improved the purification of B-512F dextransucrase to a great extent by concentrating and dialyzing the culture supernatant, treating the concentrate with dextranase, and followed by Bio-Gel A-5m column chromatography. The resulting preparation was very pure enzymatically (free of dextranase, levansucrase and sucrose phosphorylase) and had a fairly high specific activity. The

carbohydrate content, however, was relatively high and the purified enzyme had two active molecular forms.

Kobayashi and Matsuda[25] purified dextransucrase to a higher specific activity and lower carbohydrate content than Robyt and Walseth[24]. The purified dextransucrase did not contain glucosidase or dextranase. The preparation, however, acted on raffinose to produce fructose, melibiose, and a trace amount of leucrose. The authors believed that this was caused by dextran stimulation, not the presence of levansucrase or invertase. Besides, the purified dextransucrase, though homogeneous, had a molecular weight of 64 kDa, the lowest molecular weight reported for dextransucrase. This preparation, however, had very low polymerizing activity and very high sucrose hydrolyzing activity. It is possible that this protein, essentially devoid of dextran synthesizing activity, could be a subunit of the enzyme, containing only half of the two sites necessary for dextran synthesis.

More recently, Miller, Eklund and Robyt[26] have optimized the DEAE-cellulose column chromatography to purify gram quantities of dextransucrase to high specific activity (90-130 U/mg protein) and low carbohydrate content (0.2-0.7 mg/mg protein). The purified enzyme was relatively stable. When combined with an affinity chromatography step using Sephadex G-200, hundred-milligram amounts were produced with an even lower carbohydrate content (0.1 mg/mg protein) and a higher specific activity (170 U/mg protein). Levansucrase and dextranase activities were undetectable. The only drawback to this procedure was the relatively long times required, using five steps, three of which were chromatographic steps, further, the purified enzyme was not homogeneous as judged by gel-electrophoresis, which showed two major active forms of 177 and 158 kDa.

Chemical Modification of Enzymes

Of special importance in probing the active site structure of enzymes is the technique of chemical modification. In this method, a chemical reagent specific for an amino acid is used to react with the enzyme. The reagent usually reacts with and modifies the amino acid side chains in the enzyme and produces changes in some measurable property of the enzyme, such as enzyme activity or light absorbance at a specific wavelength. A correlation of modification with alteration of biological activity is established if one wants to ascribe a function to the modified residue. Usually such a correlation is established by means of a plot of biological activity versus the number of residues modified. The ideal case would be a linear relationship. If the plot gives a non-linear relationship, the number of essential residues can still be calculated by means of the statistical analysis of Tsou[27]. This method is based on a plot of the total number of modified residues against the residue activity raised to the reciprocal power of a small integer (i=1, 2, 3, etc.). Several plots can be made of the experimental data, each with a different value of i. The resulting plots are analyzed by statistical methods for the best fit to a straight line. The value of i corresponding to the best fit is then the number of residues essential for the biological activity of the protein.

Essential histidine residues in the active-site of enzymes have usually been studied by two major chemical modification methods[28]. The first method is photo-oxidation in the presence of a dye. Methylene blue and Rose Bengal are the two most commonly used dyes. Rose Bengal has generally been considered to be more specific than methylene blue[29]. The second method which is relatively specific involves reactions with diethyl pyrocarbonate for the modification of

histidine[30]. This reagent shows good specificity at neutral pH values. It carboxyethylates one of the imidazole nitrogens, and results in an increase in absorbance at 240 nm, which can serve as a good indication of histidine being modified. This monosubstitution of histidine can be reversed by reaction with hydroxyl amine resulting in the recovery of the histidine structures[30].

Numerous proteins and enzymes have been shown to have essential histidine residue(s) by this method: Bacillus amyloliquefaciens α-amylase[31], human placental aldehyde reductase II[32], mitochondrial ATPase inhibitor protein[33], amylolytic enzymes[34], and hydroxy steroid dehydrogenase[35] are only a few examples.

Acceptor Reactions of Dextransucrase

The acceptor reaction of dextransucrase has long been observed from the fact that it was able to transfer glucosyl residues from sucrose to the nonreducing end of acceptor molecules[12]. Addition of low molecular weight dextran and oligosaccharides was able to increase the amount of intermediate molecular weight dextran produced by acceptor reactions[12]. It, however, was only after 1976 that mechanism of the acceptor reaction was studied and experimental evidence presented, and a mechanism for the branching of dextran was proposed by Robyt and Taniguchi[13]. In this mechanism, a C-3 hydroxyl group on an acceptor dextran acts as a nucleophile on C-1 of the reducing end of dextranosyl-dextransucrase complex or the glucosyl-dextransucrase complex, thereby displacing dextran or glucose from the active site of dextransucrase and forming an α(1→3) branch linkage.

Later, a more detailed study of the acceptor reactions by Robyt and Walseth[14] determined the exact role that acceptor reactions play in dextran formation and in the formation of

homologous series of acceptor products. It was shown that both glucosyl and dextranosyl groups were transferred to the nonreducing ends of the exogenously added acceptors. These results indicated that acceptor reactions serve to terminate polymerization of dextran by displacing the growing dextran or glucosyl intermediate from the active-site of dextransucrase. For some acceptors, their acceptor products also serve as acceptors, and in these cases, a series of homologous acceptor products result. One such example is methyl-α-D-glucopyranoside, which gives a series of homologous acceptor products in which isomaltodextrins of varying length are attached to it by an α(1→6) linkage. For other acceptors, only single products are formed. One such example is fructose, for which only leucrose is formed.

More recently, Robyt and Eklund[36] studied the quantitative distributions of products produced from various monosaccharide and disaccharide acceptors. The relative effectiveness of the acceptors to receive the glucosyl residues from dextransucrase was measured. Maltose was found to the best acceptor of all those studied. Other acceptors in decreasing order of their effectiveness were isomaltose, nigerose, methyl-α-D-glucoside and glucose.

SECTION I:

A FACILE PURIFICATION OF *Leuconostoc mesenteroides* B-512FM DEXTRANSUCRASE

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ABSTRACT

Leuconostoc mesenteroides NRRL B-512F has been mutated by treatment with N-nitrosoguanidine. The resulting mutant (designated as B-512FM) produces 300 times as much enzyme as the parent strain. B-512FM dextransucrase in the culture supernatant was concentrated and dialyzed, and then treated extensively with Sigma crude dextranase, followed by column chromatography on Bio-Gel A-5m. The purified dextransucrase had a specific activity of 84 IU/mg and a 100-fold purification with 42% yield. SDS-gel electrophoresis of the purified enzyme showed a single protein of 158,000 which was shown to have dextransucrase activity. The purified enzyme was free of levansucrase, invertase, and glucosidase activity and retained full dextran synthesizing activity at 4°C for three months and 60% of its activity after two years. The molecular weight of 158,000 did not change during its two years of storage at 4°C. It produced the same dextran product from sucrose and the same acceptor products from α-methyl-D-glucopyranoside as the previously purified dextransucrase by DEAE-cellulose column chromatography. The 158 kDa dextransucrase was likely derived from its native form of 177 kDa in the culture supernatant by the action of a protease impurity in the dextranase. The procedure has been used to produce purified enzyme for sequencing. The molecular weight of 158,000 agrees with that calculated from its amino acid sequence.

INTRODUCTION

Dextransucrase from Leuconostoc and Streptococcus species polymerizes the glucosyl moiety of sucrose to produce dextran, an α-(1→6) linked glucan with primarily α-(1→3) linked branches. Leuconostoc mesenteroides B-512F dextransucrase has received wide attention because of the many industrial and medical uses of its dextran and modified dextrans[1]. The B-512FM dextransucrase is secreted into the culture supernatant with few contaminating enzyme activities. B-512FM dextransucrase serves as an important model in studies of the mechanism of glucan biosynthesis and its product, dextran, serves as the structural-type for dextrans.

Several reports of the purification of B-512F dextransucrase have appeared[2-6]. The method of Robyt and Walseth[3] used the B-512F strain and concentrated on the removal of contaminating enzyme activities such as levansucrase, invertase, dextranase, and sucrose phosphorylase. The method of Miller et al.[4] concentrated on obtaining relatively large amounts of enzyme with high specific activity and very low carbohydrate content. This procedure used a B-512F mutant that produced approximately 300 times as much dextransucrase as the parent B-512F strain. The purified dextransucrase, however, had two major forms with molecular weights of 158,000 and 177,000. Further, the method was time-consuming using five steps, three of which were chromatographic column steps. Kobayashi and Matsuda[5] obtained relatively small amounts of apparently homogenous dextransucrase from B-512F that had a molecular weight of 64,000, the lowest molecular weight reported for a dextransucrase. This preparation, however, had very low dextran-synthesizing activity but had relatively high sucrose hydrolyzing activity[5].

In the present procedure, we report the purification of B-

512FM dextransucrase to homogeneity with a molecular weight of 158,000 by using a fast and simple scheme of culture supernatant concentration, treatment with dextranase during dialysis, followed by gel-filtration on Bio-Gel A-5m. Dextransucrase was purified 100-fold from the culture supernatant in a 42% yield in which 99.6% of the protein and 98.9% of the carbohydrate was removed.

MATERIALS AND METHODS

Materials

L. mesenteroides B-512F was obtained from Northern Regional Research Center (Peoria, IL). Penicillium funiculosum dextranase (Crude type) was purchased from Sigma Chemical Co. (St. Louis, MO). [U-^{14}C]sucrose and [U-^{14}C-fructose]labeled-sucrose were purchased from New England Nuclear (Boston, MA). Bio-Gel A-5m, acrylamide and bisacrylamide, and molecular weight protein standards were obtained from Bio-Rad Laboratories (Richmond, CA). [U-^{14}C]dextran was prepared by using [U-^{14}C]sucrose and B-512FM dextransucrase. All other chemicals were of reagent grade and commercially available.

Methods

Mutation of L. mesenteroides B-512F

The organism was grown on the medium described by Hehre[7]. Cells from the log phase were harvested by centrifugation, washed three times with 20 mM sterile acetate buffer (pH 5.2), and then treated with N-nitrosoguanidine (150 μg/mL) for 2 hours. The treated cells were precipitated by centrifugation, washed again three times with acetate buffer, then plated onto the above medium, containing 2% agar, in petri dishes. Mutant colonies were selected for high polysaccharide production and have been designated as B-512FM. The mutant has been stable for fifteen years.

Enzyme assays

Dextransucrase activity was measured by a radioactive assay using [U-^{14}C]sucrose as previously described[4]. Levansucrase was assayed similarly except that [U-^{14}C-fructose]labeled-sucrose was used. One unit of enzyme (dex-

transucrase or levansucrase) was defined as the amount of enzyme that will incorporate 1 μmole of D-glucose or D-fructose, respectively, into polysaccharide in 1 minute. Dextranase was assayed, using [^{14}C]dextran, by measuring the disappearance of dextran. Aliquots of the dextranase digests were added to a 1.5 cm^2 pieces of Whatman 3 MM paper, which were immediately submersed in methanol, washed 3 times with methanol, dried, and counted in a liquid scintillation spectrometer, using a toluene cocktail. One unit of dextranase is defined as the amount of enzyme that will release an equivalent of 1 μmole of isomaltose in 1 minute at 37°C and pH 6.

Protein and carbohydrate determination

Protein was determined by the Lowry method[8] by using bovine serum albumin as a standard. Carbohydrate was determined by the phenol-sulfuric acid method[9] by using maltose as a standard.

Purification of dextransucrase

L. mesenteroides B-512FM was grown in a sucrose medium as described previously[4], except that Tween 80, a dextransucrase activator[10], was not added because of its possible interference with amino acid sequencing. Tween 80 (0.1% v/v) in the culture supernatant increases the enzyme activity 2- to 3-fold[10].

All of the purification steps were performed at 4°C. After removing the cells by centrifugation, the culture supernatant (1 L) was concentrated to 0.2 L with a Bio-Fiber 80 Miniplant (Bio-Rad Laboratories, Richmond, CA). Crude dextranase (180 U, 55 U/mg of protein) was added to the above culture supernatant concentrate, which was immediately dialyzed for 48 hrs against 20 L of 20 mM sodium

acetate buffer (pH 5.2). The dialysate was then concentrated to 54 mL in a dialysis tube using polyethylene glycol 20,000 as a dehydrating agent outside the dialysis tube. The concentrate (12 mL) was loaded onto a Bio-Gel A-5m column (2.5 x 60 cm), and the enzyme was eluted with 20 mM sodium acetate buffer (pH 5.2) at a flow rate of 8 mL per hour. Column fractions (3 mL) were assayed for dextransucrase, levansucrase, and protein. Fractions containing dextransucrase were pooled, concentrated in a dialysis tube with polyethylene glycol 20,000, and dialyzed against 20 mM sodium acetate buffer (pH 5.2) containing 3 mM calcium chloride. The dialysate (15 mL) was labeled as A-5m concentrate and was considered the purified enzyme.

Electrophoresis

Gel electrophoresis was performed by the method of Laemmli[11] on 5 x 90 mm cylindrical gels (7% acrylamide). SDS-Gel electrophoresis was performed on 90 x 100 mm slab gels (10% acrylamide). Protein was stained with Coomassie Blue G-250. Dextransucrase activity was detected by incubating the gels in 100 mM sucrose overnight, followed by staining for polysaccharide by a periodic acid-Schiff procedure[12].

RESULTS AND DISCUSSION

Purification of Dextransucrase

Chromatography on Bio-Gel A-5m of the dextranase-treated, culture-supernatant-concentrate is shown in Fig. 1. Dextransucrase was separated from dextranase and other proteins by gel filtration on Bio-Gel A-5m. No levansucrase activity was detected in any of the fractions. Proteins larger than dextransucrase were not found. By contrast, Robyt and Walseth have reported that the majority of the protein, including levansucrase, migrated with the void volume of the Bio-Gel A-5m column after treatment of the culture-supernatant-concentrate with dextranase (crude type)[3]. Protease impurities, in the crude dextranase used in the present study, apparently cleaved the larger proteins and levansucrase into smaller peptides, which were retarded on Bio-Gel A-5m to a greater extent than was dextransucrase. Dextransucrase was also cleaved by the protease(s) to a limited extent to form 158 kDa dextransucrase from the native 177 kDa dextransucrase. This protease hydrolysis, however, did not have a deleterious effect on dextransucrase activity or stability. The protease can be completely removed from dextransucrase by chromatography over Bio-Gel A-5m.

Table I gives the purification data for the present dextransucrase purification scheme. This simple procedure gave an overall 100-fold purification, with a dextransucrase specific activity of 84 IU/mg. The purified enzyme was obtained in 42% yield with removal of 99.6% of the protein and 98.9% of the carbohydrate. Levansucrase was non-detectable when assayed using [U-^{14}C-fructose]labeled-sucrose. The purified enzyme did not give any detectable products from raffinose, further indicating the removal of levan-

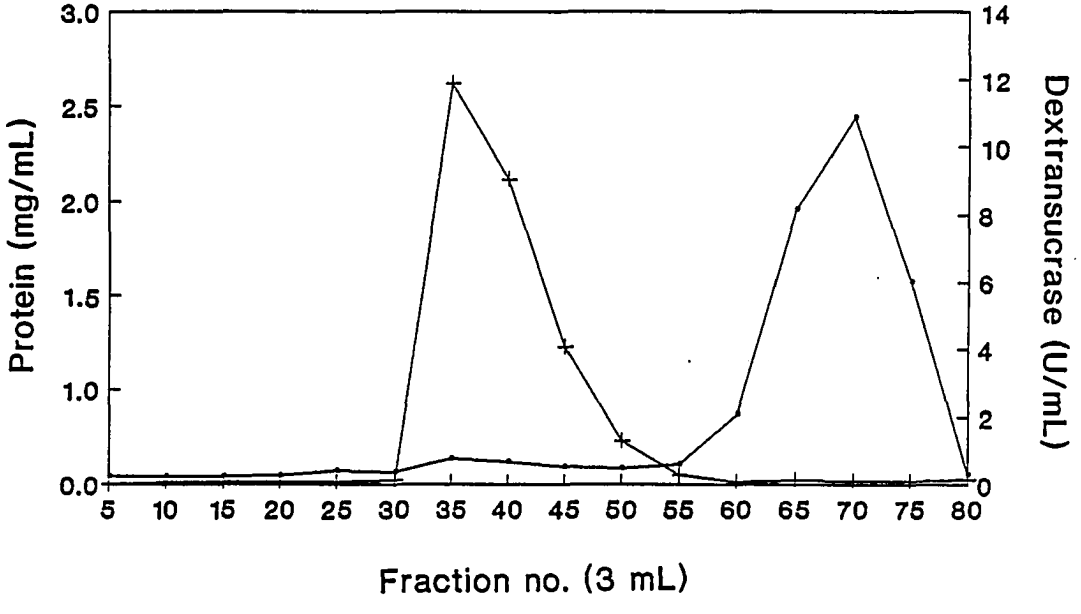


Figure 1. Chromatography of the dextranase treated, culture supernatant concentrate on Bio-Gel A-5m column: -+- dextransucrase; -•- protein

Table I. Summary of the purification of B-512FM dextransucrase

Fract.	Vol.	PF[a]	Spec. Act.	Dextransucrase		
	mL		U/mg	U/mL	total U	% yield
CS[b]	1000	1	0.84	2.6	2600	100
CSC[c]	54[d]	3	2.43	35	1890	73
A-5m[e]	68[f]	100	84.2	16	1088	42

[a]PF ---- purification factor.

[b]CS ---- culture supernatant.

[c]CSC ---- culture supernatant concentrate that was treated with dextranase and dialyzed.

[d] Addition of Tween 80 (0.1% v/v) increases the activity 2- to 3-fold.

[e]A-5m ---- pooled fractions that were concentrated.

[f]Volumes have been adjusted based on 1 L of CS.

Protein			Carbohydrate		
mg/ml	total mg	% removed	mg/ml	total mg	% removed
3.1	3100	0	7.3	7300	0
14.4	778	75	90	4896	32.9
0.19	13	99.6	1.2	82	98.9

sucrase, invertase and glucosidase[3]. The dextranase added to the culture supernatant concentrate was removed almost completely (99.95%) as measured by using [^{14}C]dextran. The trace amount of dextranase (0.0018 U/mL) in the dextransucrase preparation is insignificant. The ratio of dextranase activity to dextransucrase activity is 1 to 10,000. Polyacrylamide gel electrophoresis of the purified dextransucrase (Fig. 2), followed by activity staining, showed one major and two minor protein bands (Right Gel), all of which had dextransucrase activity (Left Gel). The major band was the native form of dextransucrase. The two minor bands were identified as aggregated forms of dextransucrase, since SDS-gel electrophoresis of the purified dextransucrase showed only one protein band (B of Fig. 3). The aggregated forms of dextransucrase were dissociated into the native monomeric form when boiled with SDS prior to the SDS-gel electrophoresis.

The amino acid sequence of B-512FM dextransucrase has been obtained for this enzyme preparation[13]. The sequence showed that the purified B-512FM dextransucrase has 1527 amino acid residues and a pI of 5.78. The relatively simple purification procedure that we report here gives homogeneous dextransucrase with a molecular weight of 158,000, relatively high specific activity, and low carbohydrate content.

Molecular Weights of Dextransucrase

SDS-gel electrophoresis of the purified dextransucrase and the supernatant is shown in Fig. 3. The purified dextransucrase showed a single protein of 158 kDa (B of Fig. 3). The culture supernatant showed a major protein band of 177 kDa and several minor protein bands of smaller molecular weights (C of Fig. 3). The purified dextransucrase (158,000) was derived from the 177,000 form found in the

Figure 2. Polyacrylamide gel electrophoresis of purified B-512FM dextransucrase: Left Gel, Coomassie Blue stain for protein; Right Gel, Periodic acid-schiff stain for dextransucrase

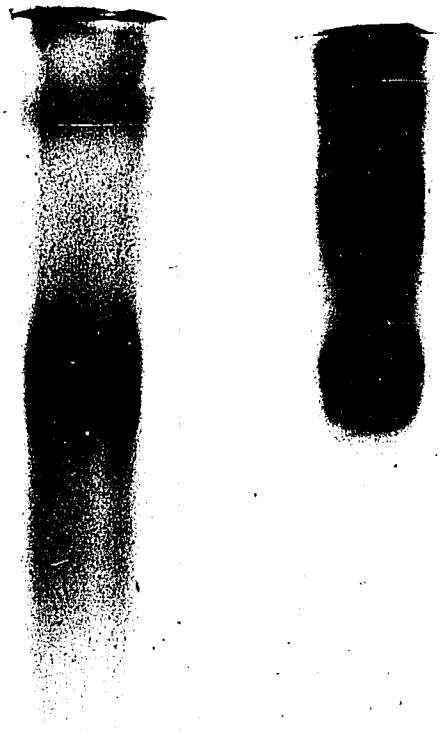
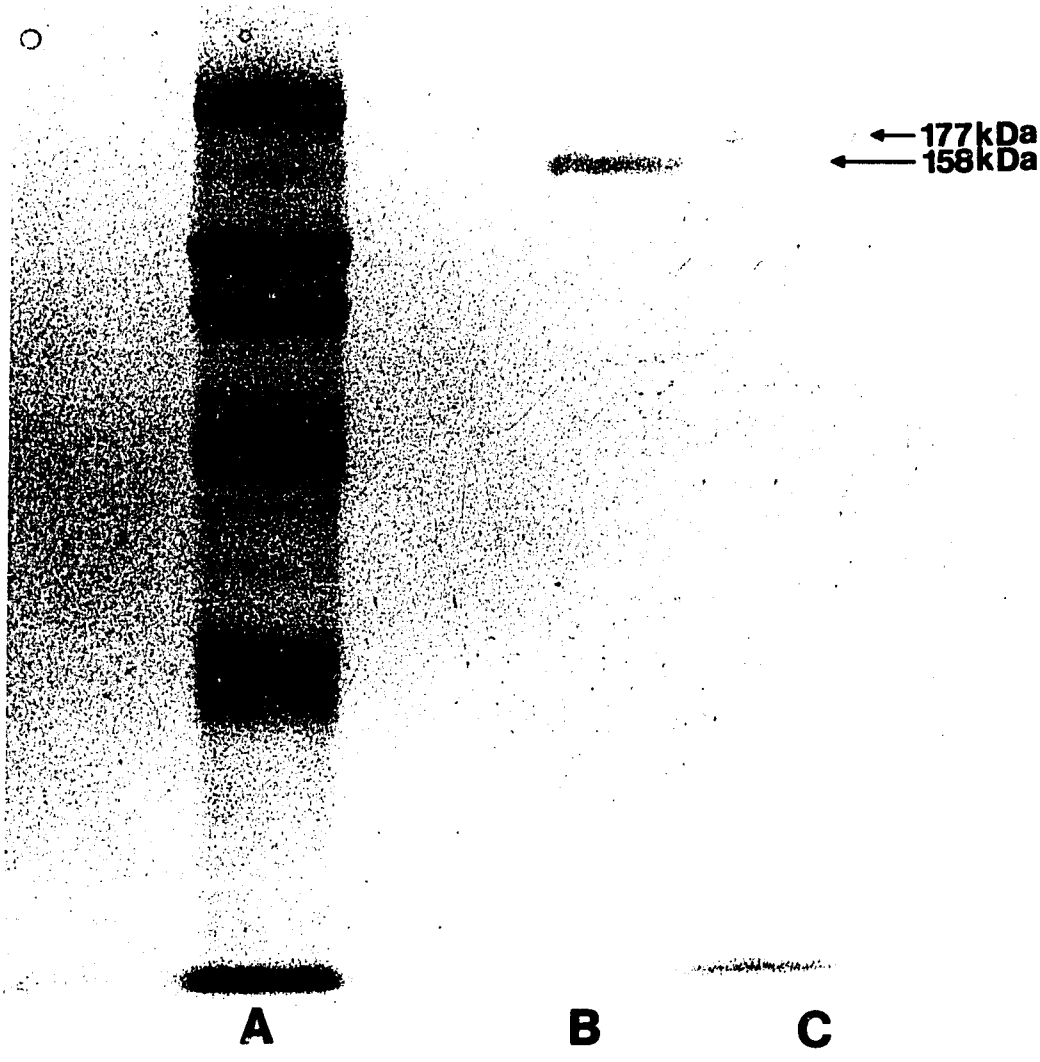


Figure 3. SDS-gel electrophoresis of purified B-512FM dextransucrase and fresh culture supernatant: Band A, protein standards (from top to bottom: myosin, ß-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin); Band B, purified dextransucrase; Band C, culture supernatant


177kDa
158kDa
A
B
C

culture supernatant by a specific proteolytic cleavage, removing 19,000 of the original peptide. The molecular weight difference in these two forms could not have been caused by dextran, since neither the addition of exogenous dextran to the purified enzyme nor the formation of endogenous dextran by the purified dextransucrase itself reacting with sucrose caused the 158,000 enzyme to increase its molecular weight.

Earlier purification procedures of L. mesenteroides B-512F dextransucrase have failed to give a single protein[3,4]. Miller et al.[4] have purified dextransucrase to very high specific activity (90-170 IU/mg protein) with very low carbohydrate content (1-100 μg/mg protein). The purified enzyme, however, always had two major molecular forms of 177 kDa and 158 kDa. During its storage, the 158 kDa form usually increased at the expense of the 177 kDa form, apparently due to an endogenous protease activity in the L. mesenteroides B-512FM culture supernatant that was copurified with dextransucrase.

Kobayashi and Matsuda[5] reported a B-512F dextransucrase of a single peptide of 64 kDa. This preparation, however, had very low dextran synthesizing activity and very high sucrose hydrolyzing activity. It is possible that this small protein, essentially devoid of dextran-synthesizing activity, could be a subunit of the enzyme, containing only half of the two sites necessary for dextran synthesis[14].

The multiplicity of dextransucrase molecular forms caused by proteolysis has also been reported for glucansucrases from Streptococci species. Russell[15] found three forms of glucansucrase (132, 140 and 150 kDa) from Streptococcus mutans 3209 in the presence of the protease inhibitor phenyl-methyl sulfonyl fluoride, but only two forms (132 and 140 kDa) in its absence. It seems that the 150 kDa peptide

was cleaved by proteases to give 132 and 140 kDa. Kenney and Cole[16] have reported that a 138 kDa glucansucrase from S. mutans 3209 was derived from proteolysis of a 162 kDa form. Grahame and Mayer[17] found that dextransucrase from S. sanguis was initially secreted as a 174 kDa form, which was then converted to 156 kDa form by proteolysis, very similar to what we observed for L. mesenteroides B-512FM dextransucrase when crude dextranase is added.

Character of the Purified Dextransucrase

The purified B-512FM dextransucrase had essentially identical catalytic properties as the previously purified B-512F dextransucrase[3]. The dextran produced by the purified enzyme was a soluble, high molecular weight polysaccharide with 96% α-(1→6) linkages and 4% α-(1→3) branch linkages as determined by dextranase digestion of a ^{14}C-labeled sample that had been synthesized by the enzyme. When α-methyl-D-glucopyranoside was added to a sucrose-dextransucrase digest, a characteristic homologous series of α-methyl isomaltodextrins[18] were observed as acceptor products.

The purified enzyme retained all of its activity when stored at 4° for 3 months and 60% of its activity after 2 years of storage, and its molecular weight remained 158,000. No precipitate was formed in the enzyme solution during its storage at 4°C.

Aggregation of Dextransucrase

Under non-denaturing conditions, glucansucrases usually aggregate strongly[3,19]. The purified B-512FM dextransucrase was found to form aggregates, but only to a very minor extent. Even after two years of storage at 4°, the major portion of the purified enzyme retained its 158 kDa form as shown by the major protein band with dextransucrase activity

in Fig. 2. Only a small portion of the purified enzyme formed aggregates as shown by the two minor protein bands with dextransucrase activity (Fig. 2). By contrast, Robyt and Walseth[3] found that B-512F dextransucrase, prior to dextranase treatment, migrated with the void volume of Bio-Gel A-5m column. This indicated that all of the enzyme was aggregating into large molecular units. Studies by Kobayashi and Matsuda[19] also indicated that the B-512F dextransucrase exists almost entirely in large aggregated forms, in which the aggregates were a mixture of oligomers of a 65 kDa promotor and their charge isomers. Aggregation of glucansucrase from Streptococci have also been observed. Luzio et al.[20] found that dextransucrase from S. sanguis forms aggregates of very large dimensions, and the addition of SDS and nonionic detergents caused dissociation of these aggregates into smaller sizes. Newman et al.[21] found that 1.55 M ammonium sulfate converted part of a 100-110 kDa glucansucrase from S. mutans into a high molecular weight aggregate.

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SECTION II:

ESSENTIAL HISTIDINES IN DEXTRANSUCRASE: BY CHEMICAL MODIFICATION AND PHOTO-DYE-OXIDATION

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ABSTRACT

Treatment of Leuconostoc mesenteroides B-512FM dextransucrase by diethylpyrocarbonate (DEP) at pH 6.0 and 25° or photo-oxidation in the presence of Rose Bengal or methylene blue dyes at pH 6.0 and 25° caused a rapid decrease of enzyme activity. Both types of inactivations followed pseudo-first-order kinetics. Enzyme partially inactivated by DEP could be reactivated completely by treatment with 100 mM hydroxylamine at pH 7 and 4°. The presence of dextran partially protected the enzyme from inactivation. At pH 7 or below, DEP is relatively specific for the modification of histidine. DEP-modified enzyme showed an increased absorbance at 240 nm, indicating the presence of ethoxyformylated histidine residues. DEP modification of the sulfhydryl group of cysteine and the phenolic group of tyrosine was ruled out by showing that native and DEP-modified enzymes had the same number of sulfhydryl and phenolic groups. DEP modification of the ϵ-amino group of lysine was ruled out by reaction at pH 6 and reactivation with hydroxylamine, which has no effect on DEP modified ϵ-amino groups. The photo-oxidized enzyme showed a characteristic increase in absorbance at 250 nm, also indicating that histidine was being oxidized, and no decrease in the absorbance at 280 nm, indicating that tyrosine and tryptophan were not oxidized. A statistical kinetic analysis of the inactivation data by DEP showed that two histidine residues are essential for the enzyme activity. Previously it was proposed that two nucleophiles at the active site attack bound sucrose to give two covalent glucosyl-enzyme intermediates. We now propose that in addition, two imidazolium groups of histidine at the active site donate protons to the leaving fructose moieties. The resulting imidazole groups then facilitate the formation

of the α-1→6 glycosidic linkage by abstracting protons from the C_6-OH groups and become reprotonated for the next series of reactions.

INTRODUCTION

Dextransucrase from Leuconostoc mesenteroides B-512FM catalyzes the polymerization of the glucosyl moiety of sucrose to form dextran with the release of fructose. This enzyme has been the subject of several mechanistic studies in this laboratory[1-3]. Robyt et al.[1] have proposed a mechanism for dextran synthesis, in which two nucleophiles at the active site attack two bound sucrose molecules to give two glucosyl units, covalently linked to the nucleophiles through C-1. In this process, two protons are donated to the leaving fructose moieties[3]. The nature of the proton donors, however, is not known. Studies of the pH dependence of dextransucrase activity suggested that imidazole moiety of histidine residue may serve as the proton donor[4]. Since the pKa value of an amino acid residue in a protein depends on its micro-environment, results from pH studies are usually not conclusive. We have, thus, studied the question by chemically modifying dextransucrase with DEP and dye-photooxidation to further elaborate the mechanism of dextransucrase.

DEP has been widely used in modifications of various enzymes[5]. This modification is based on DEP carbethoxylation of the side chains of amino acid residues in proteins, especially the imidazole group of histidine[5]. At suitable pH values, DEP can react with other amino acid side-chain groups as well, such as the hydroxyl group of tyrosine, the sulfhydryl group of cysteine, and the ϵ-amino group of lysine[6]. However, at pH 7 or below, DEP specifically reacts with the imidazole group of histidine[5]. Essential histidine residues in sheep liver 6-phosphogluconate dehydrogenase[7], Bacillus amyloliquefaciens α-amylase[8], amylolytic enzymes[9], and bovine heart succinate dehydrogenase[10] have been speci-

fically modified with this reagent.

It is also known that amino acids such as histidine, tryptophan, methionine, and tyrosine in a protein can be photo-oxidized in the presence of a suitable photosensitizing dye. The photo-oxidation of histidine residues in enzymes has been widely used in determining the essential role of imidazole group in the action of many enzymes[11,12]. Two dyes, methylene blue and Rose Bengal, have been most widely used to photosensitize the oxidation of histidine residues of proteins.

Studies[13,14] have shown that Rose Bengal is more selective for the photo-oxidation of histidine than is methylene blue. Both types of photo-oxidations of histidine produce an increase in absorbance at 250 nm[7,15,16]. In this study, we have chemically modified dextransucrase with DEP at pH 6.0, and have photo-oxidized this enzyme in the presence of methylene blue and Rose Bengal. We present evidence that two histidine residues are essential for the activity of dextransucrase from Leuconostoc mesenteroides B-512FM.

MATERIALS AND METHODS

Materials

DEP, L-histidine, N^{α}-acetyl-L-histidine, N-acetyl-imidazole, 5,5'-dithiobis(2-nitrobenzoic acid) and dextran T10 and T500 were purchased from Sigma Chemical Company (St. Louis, MO). Sucrose, hydroxylamine hydrochloride, and Rose Bengal dye were purchased from Fisher Scientific Company (Fairlawn, NJ). Methylene blue chloride was purchased from Hartman Leddon Co. [U-^{14}C]sucrose was purchased from ICN Pharmaceutical, Inc. All other chemicals were of analytical grade, and commercially available.

Dextransucrase from Leuconostoc mesenteroides NRRL B-512FM was purified up through the stage of DEAE-cellulose chromatography as reported previously[17], and had a specific activity of up to 70 IU/mg.

Methods

Enzyme assay

Dextransucrase activity was assayed radiometrically using [U-^{14}C]sucrose as previously described[17].

Protein and carbohydrates analysis

Protein was measured by the procedure of Lowry et al.[18] Carbohydrate was measured by the phenol-sulfuric acid method[19].

Chemical modification with DEP

The enzyme solution (0.3-1.0 mg protein/ml) in 100 mM phosphate buffer, pH 6.0, was incubated at 25° with DEP at several concentrations between 2.8 mM and 30 mM. The concentration of ethanol in the reaction mixture of the enzyme did not exceed 5% (w/v), and was not found to have a notice-

able effect on the enzyme stability or activity. The reaction with DEP was stopped at different times by transferring one part of an aliquot of the reaction mixture into nine parts of 100 mM phosphate buffer, pH 6.0, containing 20 mM L-histidine. The enzyme activity was then assayed at 25° and pH 5.2. When the absorbance spectrum of the modified protein was measured, the L-histidine was omitted. The time course of carbethoxylation of imidazole groups was followed continuously by recording the change in absorbance at 240 nm.

The concentration of DEP in the stock solution was determined by measuring the increase in absorbance at 240 nm when an aliquot of diluted DEP was added to 10 mM N^{α}-acetyl-L--histidine in 50 mM acetate buffer, pH 6.0[20]. The concentration of DEP was calculated by using an extinction coefficient of 3.2×10^3 M^{-1} cm^{-1} at 240 nm[21].

Protection of enzyme against modification

Dextransucrase (0.075 mg protein/ml, 70 units/mg) in 50 mM phosphate buffer (pH 6.0) was incubated with 5 mg/ml of dextran T10 or T500 for 20 minutes at 25°. The enzyme was then modified with 14.5 mM DEP. A control was prepared similarly except no dextran was preincubated with enzyme before modification. The activity was assayed without removal of dextran, which has no effect on the assays[22].

Reactivation of modified enzyme

Dextransucrase was modified with 2.8, 5.8, and 11.5 mM of DEP for 0.5 hour. Reversal of the modification was achieved by adding hydroxylamine (pH 7.0) to the modified enzyme (and ethanol treated control) to a final concentration of 100 mM in 100 mM phosphate buffer, pH 7.0, and incubation at 4° for 4 hours. The excess hydroxylamine was then removed by

dialysis against 25 mM acetate buffer (pH 5.2) at 4° for 20 hours, before enzyme activity was assayed.

Photo-oxidation of dextransucrase

Dextransucrase (0.5 mg protein/ml and 16 IU/ml) in 20 ml of 50 mM acetate buffer (pH 5.2) was photo-oxidized with 40 µg/ml Rose Bengal or methylene blue dye by irradiation with a 200 W floodlamp held 20 cm above the sample surface. The reaction was stirred and the temperature was controlled at 25°C with a circulating water bath. Aliquots (0.5 ml) were removed at various times between 1 and 7 hours and placed in the dark. Enzyme activity of these samples was assayed without removal of excess dye, which was not found to affect the enzyme activity. Controls were treated similarly either with 40 µg/ml of dye in the dark, or irradiated in the absence of dye. Spectra of both native and photo-oxidized enzyme were obtained in 30 mM Tris-HCl buffer (pH 8.0). Methylene blue dye was removed from both the photo-oxidized enzyme and the control by chromatography on Bio-gel P-30 before the spectra were taken.

RESULTS

Chemical Modification with DEP

Treatment of dextransucrase with DEP of 11.5, 19.3, and 27 mM led to loss of enzyme activity. Plots of log residual activity versus the time of incubation at three concentrations of inhibitor were linear, indicating the inactivation followed pseudo-first-order kinetics (Fig. 1). A plot of the observed pseudo-first-order rate constants against the concentrations of DEP gave a straight line passing through the origin (inset Fig. 1), from which a second-order rate constant of 2.65 $M^{-1}Min^{-1}$ was obtained. The DEP modification led to an increase in absorbance at 240 nm, which was proportional to the degree of inactivation (Fig. 2).

Protection against DEP modification

Dextransucrase, preincubated with dextran T10 or T500, lost 74% and 65% of its original activity respectively when reacted with 14.5 mM DEP, while enzyme in the absence of dextran lost 91% of its original activity (Fig. 3). This result shows that product dextran, which is known to bind at the active site as an acceptor[2], protects dextransucrase from modification by DEP.

Reactivation of DEP Modified Enzyme

Modified dextransucrase with 20 to 80% of the original activity regained 40% to 100% of the activity when treated with 100 mM hydroxylamine in 100 mM phosphate buffer (pH 7.0) (Table 1).

Specificity of DEP Inactivation

Although DEP at pH 6.0 reacts with the side chain of histidine residues with considerable specificity, possible

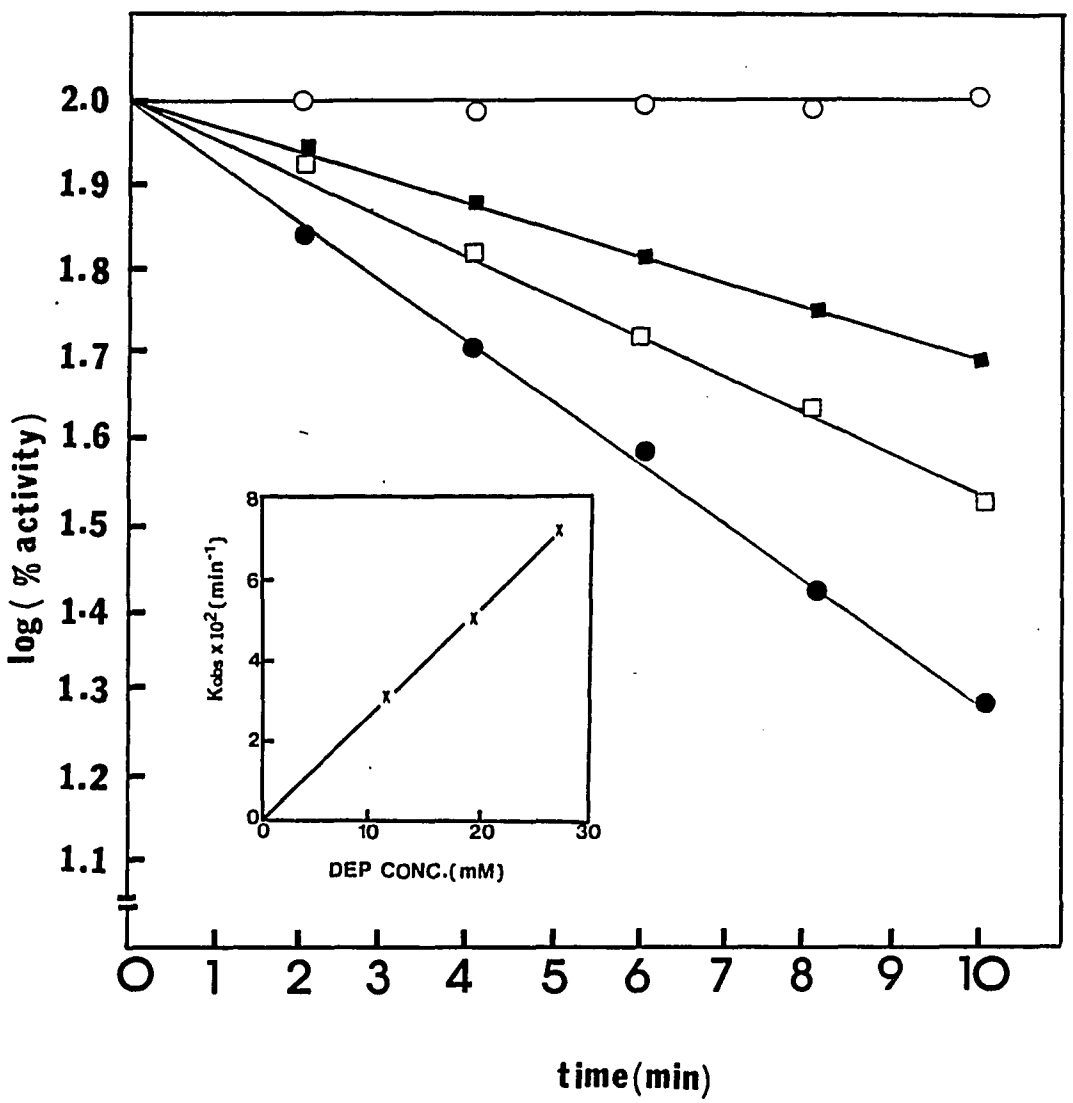

Figure 1. Kinetics of inactivation of dextransucrase by DEP of 0 mM (o), 11.5 mM (■), 19.3 mM (□), and 27 mM (●). Inset: A plot of observed pseudo-first-order rate constants vs. initial DEP concentrations

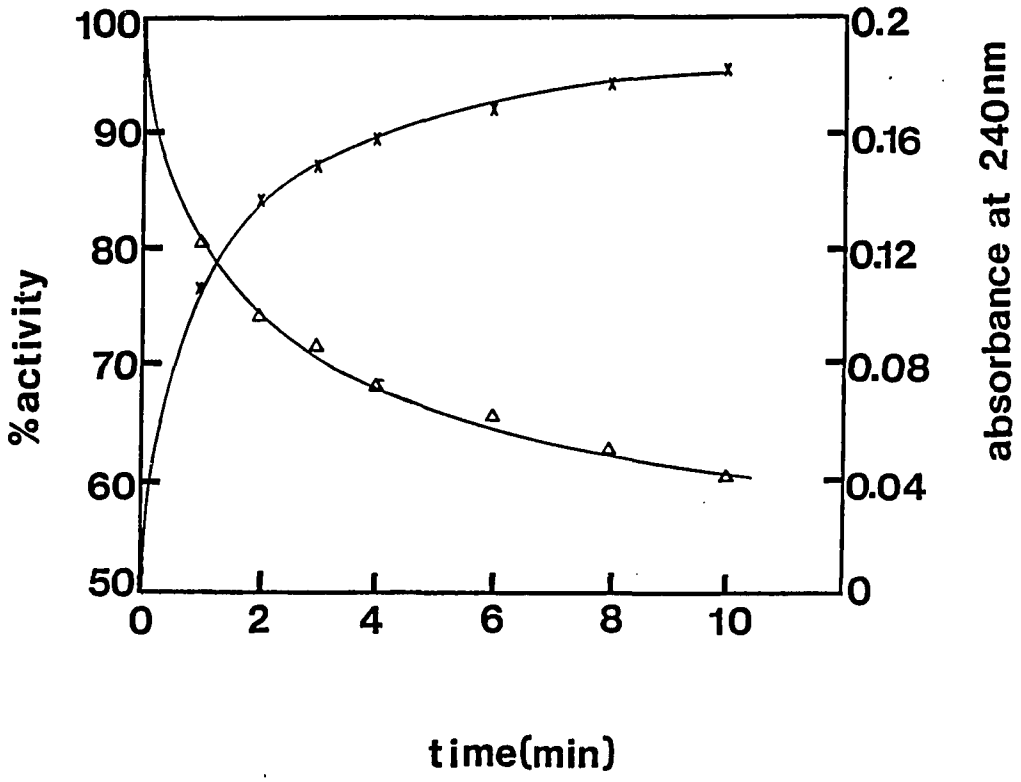

Figure 2. Correlation of DEP inactivation of dextransucrase by 17 mM DEP (Δ) with the increase in absorbance (x) at 240 nm

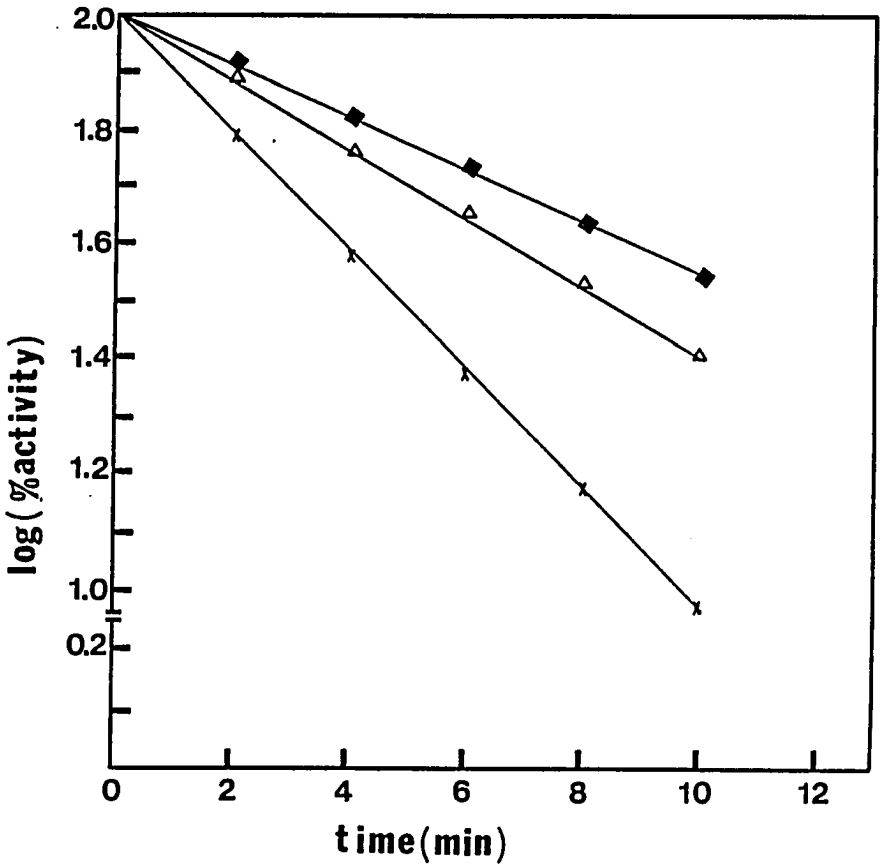

Figure 3. Protection of DEP modification of dextransucrase by no dextran(x), 5 mg/ml of dextran T10 (Δ) and 5 mg/ml of dextran T500 (◆)

Table 1. Reactivation of modified dextransucrase by hydroxylamine

DEP (mM)	% Activity before	% Activity after
0.0	100	100
2.8	77	100
5.8	62	95
11.5	29	42

modification of other residues were studied. Sulfhydryl groups in dextransucrase were titrated at pH 8.1 with 0.3 mM 5,5'-dithiobis (2-nitrobenzoic acid)[23]. The native enzyme gave a value of 1.1 sulfhydryl groups per enzyme molecule for a molecular weight of 158,000[24]. The DEP modified enzyme with 10% of its original activity gave a value of 1.0. Therefore, it was concluded that sulfhydryl groups were not being modified and were not the cause of enzyme inactivation. Since lysine can only be modified by DEP at pH values higher than 7.0, and ethoxyformylated lysine residues cannot be de-ethoxyformylated by neutral hydroxylamine[7], the pH of the reaction and the restoration of activity by hydroxylamine rules out the possibility of lysine modification. The tyrosine residue modification can also be excluded, since there was no decrease in absorbance at 278 nm when dextransucrase was completely inactivated by DEP. The modification of tyrosine residues with N-acetylimidazole at pH 7.5 showed that both native and modified enzymes had an identical decrease in absorbance at 278 nm (ascribable to the O-acetylation of tyrosine residues)[25]. This result further excludes the tyrosine modification by DEP. All of these results taken together with the proportional increase in absorbance at 240 nm with the inactivation of the enzyme and the relatively high specificity of DEP reaction at pH 6 for imidazole groups indicated that the inactivation of B-512FM dextransucrase by DEP is due to the modification of the imidazole ring of histidine residues.

Number of Essential Histidine Residues

Inactivation of dextransucrase by DEP was correlated with the number of histidine residues modified (Fig. 4). The plot is nonlinear, indicating that not all histidine residues are modified at the same rate. Some histidine residues

are more accessible for modification than others. Extrapolation of the first phase of the plot to zero enzyme activity showed that 14 histidine residues were modified out of a total of approximately 40 histidine residues per 158 Kd. Such an extrapolation does not give the number of histidines that are essential for the enzyme activity, it rather suggests that these 14 histidine residues are modified at significantly but not markedly different rate than the other histidine residues. Tsou[26] has shown that the number of essential residues, modified under conditions, where both essential and nonessential residues are modified at significantly but not markedly different rates, can be determined by correlating the rate of loss of enzyme activity with the rate of modification of the total number of reactive residues by Equation 1:

$$m = n(1-x) = n-p(A/A_0)^{1/i} - (n-p)(A/A_0)^{\alpha/i} \qquad (1)$$

where m is the number of histidine residues modified, n is the total number of histidine residues modified to give complete loss of enzyme activity, p is the total number of histidine residues including i essential residues modified at rate constant K_1, n-p is the number of histidine residues modified at rate constant K_2 ($K_2 = \alpha K_1$), A/A_0 is the fraction of enzyme activity at any time during the modification, and x is the total fraction of unmodified residues remaining at any A/A_0. Equation 1 can be rearranged to give Equation 2:

$$\log[nx/(A/A_0)^{1/i} - p] = (\alpha-1)/i\log(A/A_0) + \log(n-p) \qquad (2)$$

$\log[nx/(A/A_0)^{1/i} - p]$ was plotted against $\log(A/A_0)$ by using the values of n = 40, p = 14, and i = 1, 2, 3, 4, 5, and 6. Linear regression variances (R^2) were 0.81, 0.94, and 0.93

for i = 1, 2, and 3; and were significantly smaller than 0.9 when i = 4, 5, and 6. When i = 2 we obtained the best linear fit to the equation, with a linear regression variance $R^2 = 0.94$. This result suggested that two histidine residues per 158 Kda molecular weight[17] are essential for enzyme activity.

Photo-Dye Oxidation

Irradiation of dextransucrase at 25° in the presence of methylene blue or Rose Bengal led to loss of enzyme activity. Inactivations with either methylene blue or Rose Bengal were dependent on the dye concentration (Figs. 5 and 6). The loss of activity was linear when log % residual activity was plotted versus time, indicating pseudo-first-order kinetics.

Dextransucrase (0.6 mg protein/ml), photo-oxidized in 30 mM Tris-HCl buffer (pH 8.0) in the presence of 80 μg/ml of methylene blue, had only 3% of its original activity. The photo-oxidized enzyme, after removal of methylene blue by chromatography on Bio-gel P-30, showed an increase in absorbance at 250 nm (Fig. 7), indicative of the modification of the imidazole ring of histidine[27]. Since no decrease in absorbance at 278 nm was observed, tyrosine or tryptophan involvement in this process can be excluded.

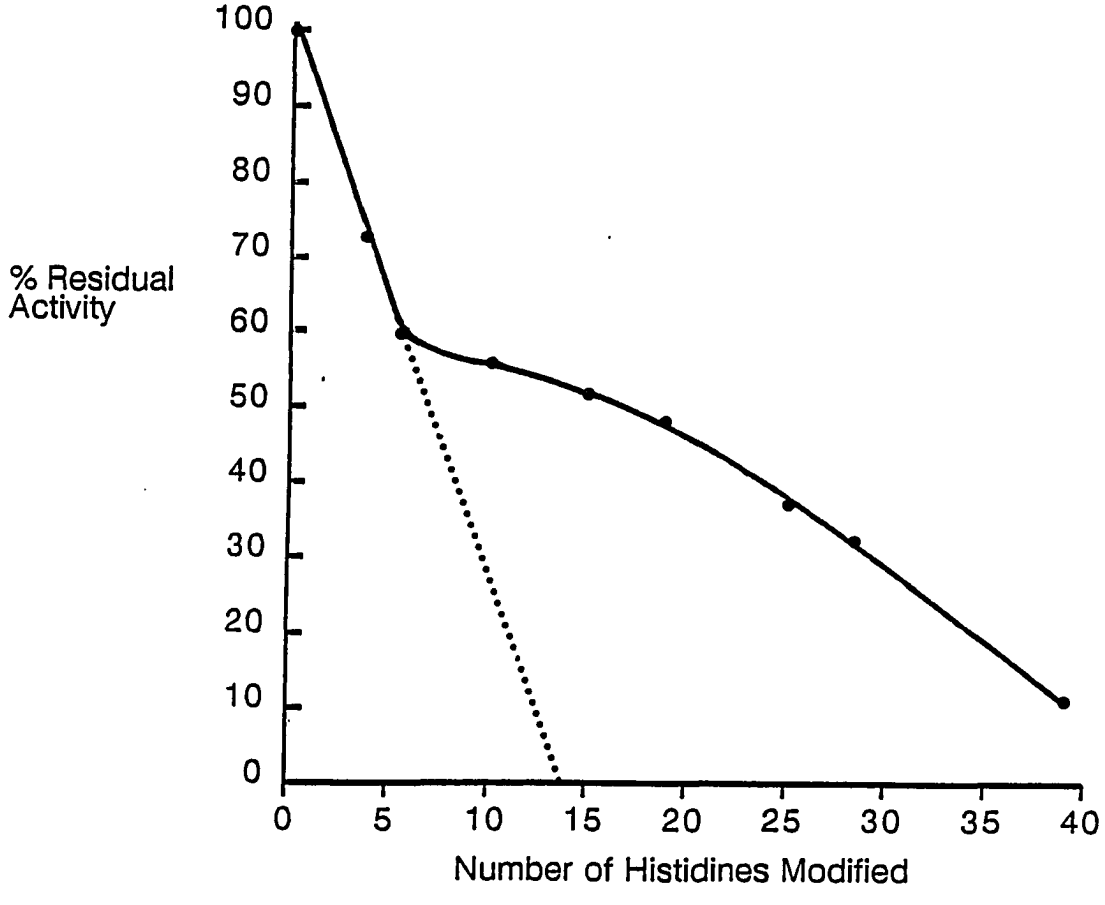

Figure 4. Correlation of the fraction of enzyme activity remaining with the number of histidine modified

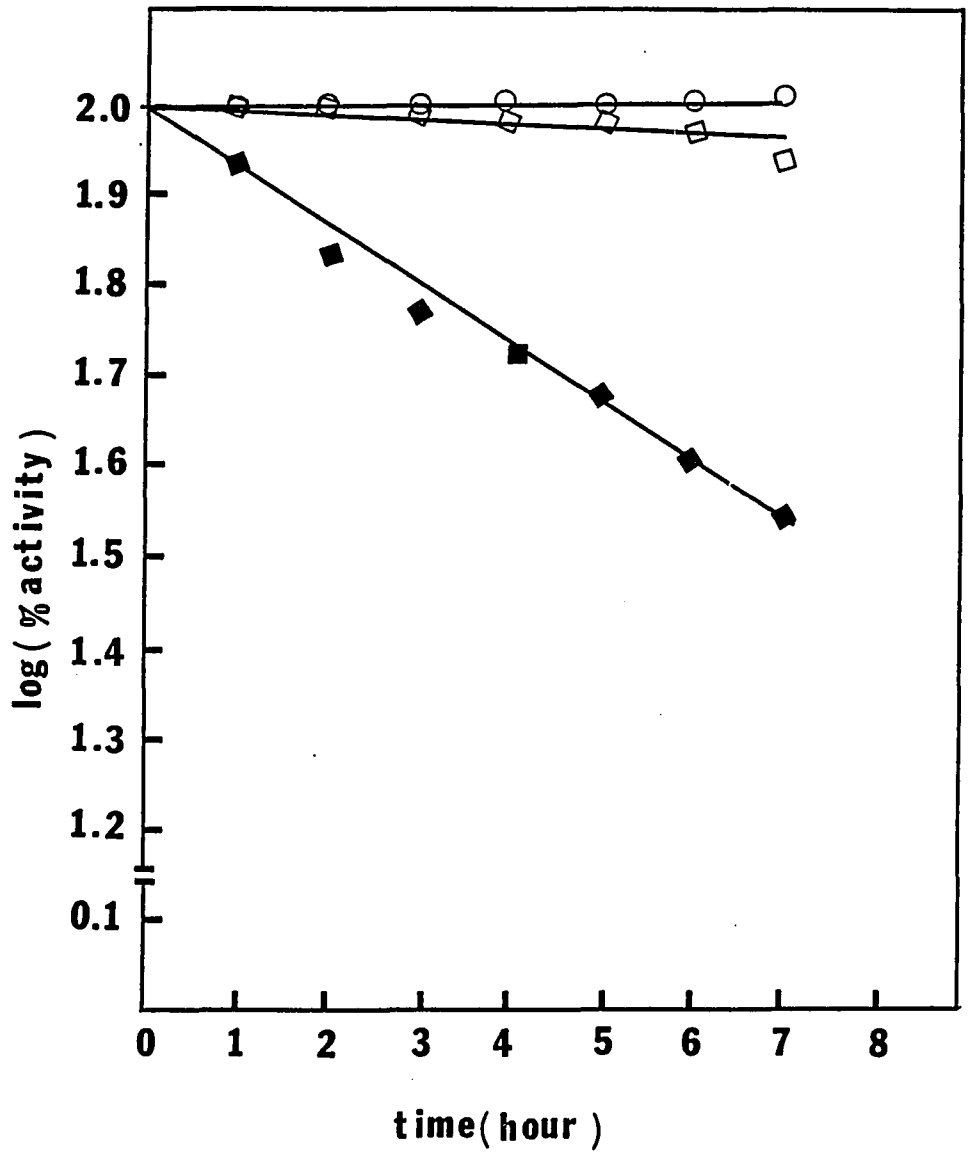

Figure 5. Time course of photo-oxidation of dextransucrase with 40 μg/ml of methylene blue (◆). Two controls were prepared similarly with dye in the dark (o), or without dye in the light (◇)

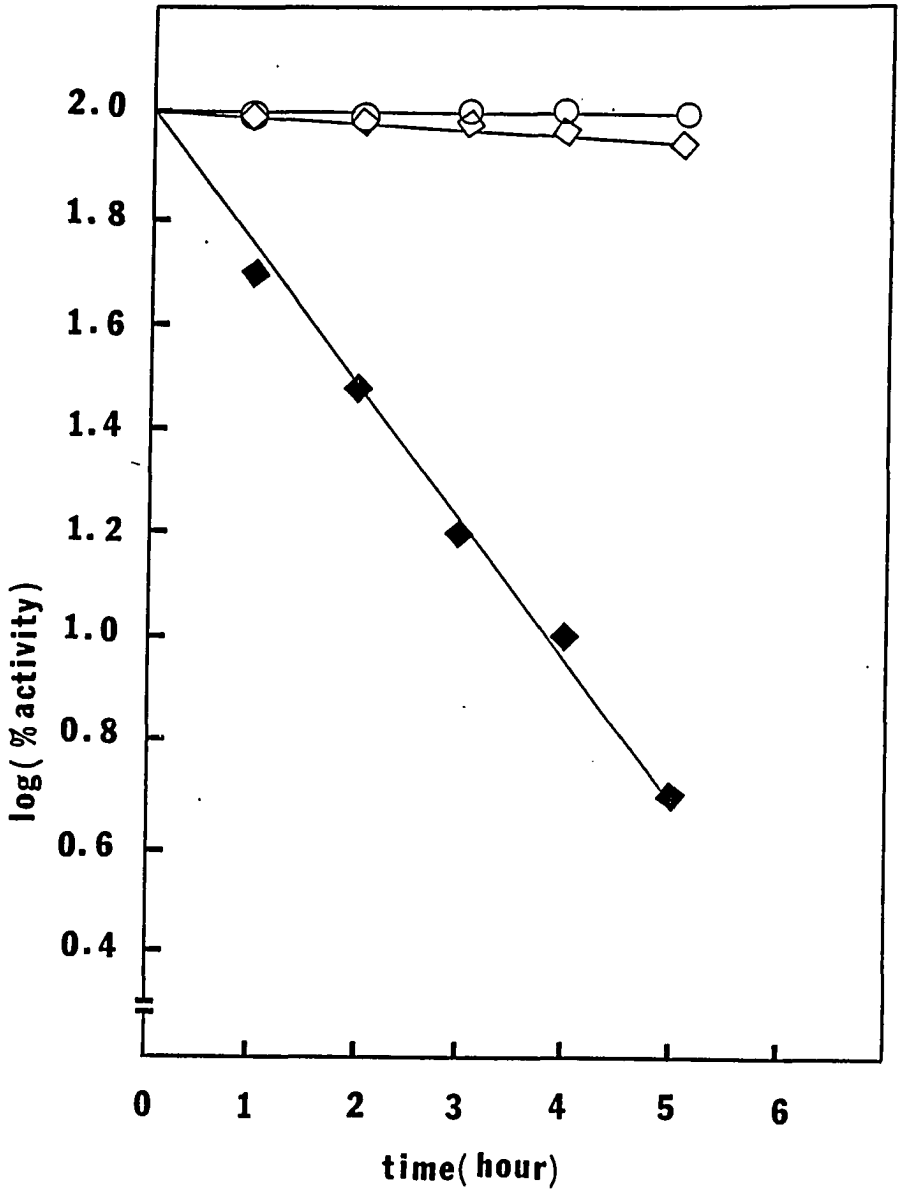

Figure 6. Time course of photo-oxidation of dextransucrase with 40 μg/ml of Rose Bengal (◆). Two controls were prepared similarly with dye in the dark (○), or without dye in the light (◇)

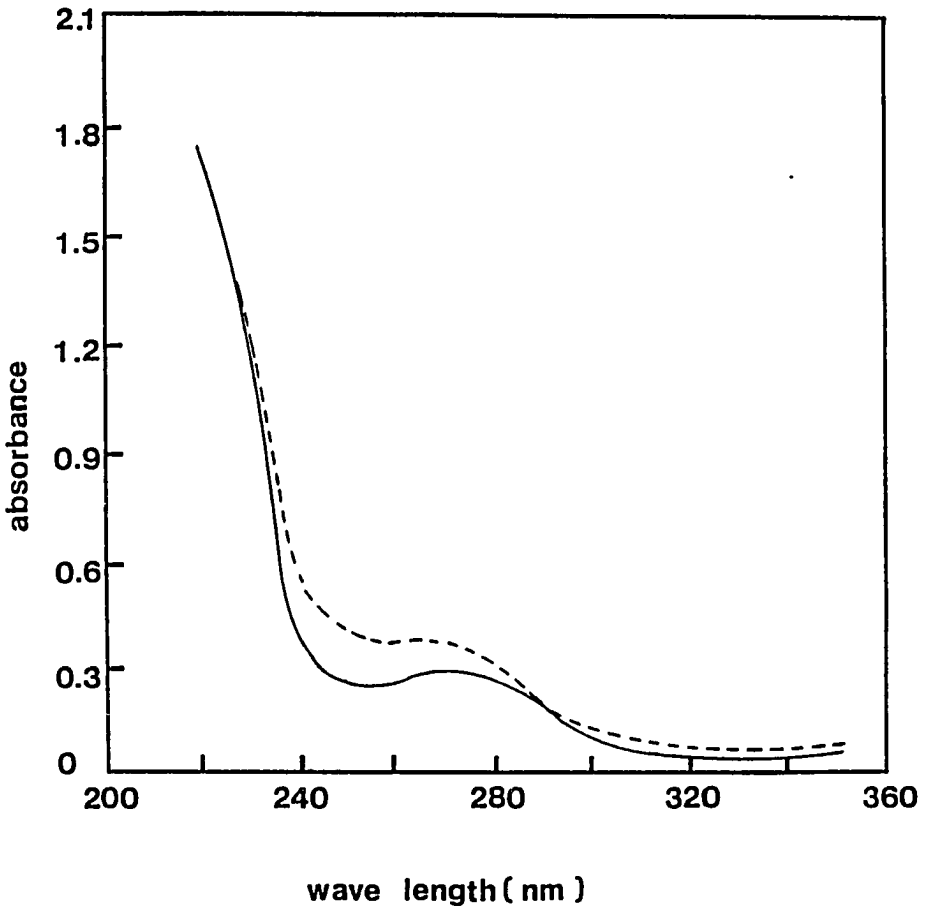

Figure 7. Absorption spectra of the native (solid line) and photo-oxidized (broken line) dextransucrases

DISCUSSION

Robyt and Eklund[3] have postulated the role of proton donors at the active site of dextransucrase. The nature of these proton donors, however, is not known. The pH dependence of kinetic parameters have suggested the likely involvement of imidazole groups of histidine[4]. Involvement of the imidazole groups at the active site was proposed by Neely[28], who showed that the irradiation of dextransucrase in the presence of methylene blue caused loss of enzyme activity. pH studies are only presumptive for identifying groups at the active site as the pKa values of amino acid residues of proteins can vary widely in different microenvironments. Photo-oxidation in the presence of methylene blue could also destroy other amino acid residues besides histidine. Therefore, we have studied the chemical modification of dextransucrase with DEP and photo-oxidation with Rose Bengal, a dye that has a higher specificity for oxidizing histidine than does methylene blue.

DEP can react with several amino acid side chain groups. It has been reported, however, that at pH 7 or below, DEP specifically ethoxyformylates the imidazole group of histidine[5,10]. We treated dextransucrase with DEP at pH 6.0 and found that dextransucrase was inactivated. The inactivation at three concentrations of DEP followed pseudo-first-order kinetics, which indicated a direct correlation between the inactivation and the modification of dextransucrase. A plot of observed pseudo-first-order rate constants against DEP concentrations showed a straight line passing through the origin. After modification with DEP, there was a characteristic increase in the absorbance at 240 nm, which was proportional to the degree of inactivation. This indicated that N-carbethoxylhistidine residues were being formed. The

characteristic increase in absorbance at 240 nm enabled us to correlate the degree of inactivation to the number of histidines modified. Treatment of this kinetic data of inactivation using Tsou's statistical method[26] suggested the presence of two histidine residues essential for enzyme activity. The role of these two essential histidine residues as proton donors is discussed below.

Because dextran is the product of the action of dextransucrase and can undergo acceptor reactions to give the formation of α-1→3 branch glycosidic linkages[2], dextran binds at the active site. In the presence of dextrans of different molecular weights, the enzyme was protected from DEP inactivation by binding at the active site, supporting the conclusion that histidine residues undergoing modification participate as catalytic groups at the active site of dextransucrase. We also tried sucrose to protect dextransucrase from inactivation, but no conclusion was possible as sucrose was rapidly converted into dextran by the enzyme. When dextransucrase was inactivated 23% by DEP, addition of 100 mM hydroxylamine completely restored its activity. The restoration of dextransucrase activity by hydroxylamine further suggests that the imidazole group of histidine is being ethoxyformylated by DEP, as it is known that hydroxylamine can remove the ethoxylformyl group from imidazole[5]. It is also known that prolonged incubation with excess of DEP can result in the formation of diethoxyformylated histidine derivatives[5]. Treatment of the disubstituted derivative with hydroxylamine opens the imidazole ring and destroys the histidine side chain. Our results (Table 1) showed that DEP-modified enzyme with 62% and 29% of the original activity regained 95% and 40% of their original activity respectively upon treatment with hydroxylamine. The incomplete reactivation of the more highly inactivated

dextransucrase is most probably due to the formation of the diethoxylformyl derivative. The reactivation of DEP modified enzyme upon treatment with hydroxylamine ruled out the possibility of lysine involvement as DEP modified lysine can not be reversed by hydroxylamine[7]. Chemical analysis with 5,5'-dithiobis (2-nitrobenzoic acid) provided evidence that cysteine was not modified by DEP. Further, the proportional increase in absorbance of the protein at 240 nm indicates that the imidazole group of histidine was modified.

Dextransucrase was inactivated by photo-oxidation in the presence of either methylene blue or Rose Bengal. Both inactivations followed pseudo-first-order kinetics and the oxidized enzyme showed an increase in absorbance at 250 nm, indicating that histidine residues were photo-oxidized[7,27].

Taken together, the following results show that imidazole groups at the active site of dextransucrase are involved in catalysis: pseudo-first-order inactivation by DEP and the consequent proportional increase in the protein absorbance at 240 nm; the reversal of the DEP inactivation by hydroxylamine; protection of DEP inactivation by dextran; and the pseudo-first-order inactivation by dye photo-oxidation and the consequent increase in the absorbance at 250 nm.

DEP has been used over a wide range of concentrations from 0.01 mM to 40 mM (the maximum solubility in water)[5]. In the present investigation, the concentration of DEP needed for the inactivation of dextransucrase was higher than for some other proteins. This could reflect the lesser accessibility of the histidyl residues at the active site, which might be wholly or partially due to the presence of covalently bound dextran at the active site.

In the synthesis of dextran by dextransucrase, it has been proposed[1,3] that two nucleophiles (X^-) at the active site attack two bound sucrose molecules to give two glucosyl

units covalently linked to the nucleophiles through carbon-1. In this process, we propose that two imidazolium groups of histidine residues at the active site donate protons to the oxygen of the leaving fructose moieties (step 1 of Fig. 8). The imidazolium ions then are changed to imidazole groups, and then one of the imidazole groups abstracts the proton from the C_6-hydroxyl group of one of the covalently linked glucosyl units, facilitating the C_6-hydroxyl group nucleophilic attack on C-1 of the other glucosyl unit to form the α-1→6 glycosidic bond (step 2 of Fig. 8). As the glycosidic linkage is formed, the X-group attached to the leaving glucose moiety is released and becomes free to attack another sucrose molecule. The protonated imidazole group that abstracted the proton from the C_6-OH group to facilitate the formation of the glycosidic linkage now transfers its proton to the leaving fructosyl moiety. This process is then repeated back and forth between the two X-groups and the two imidazolium and imidazole groups to give the synthesis of dextran as shown in Figure 9.

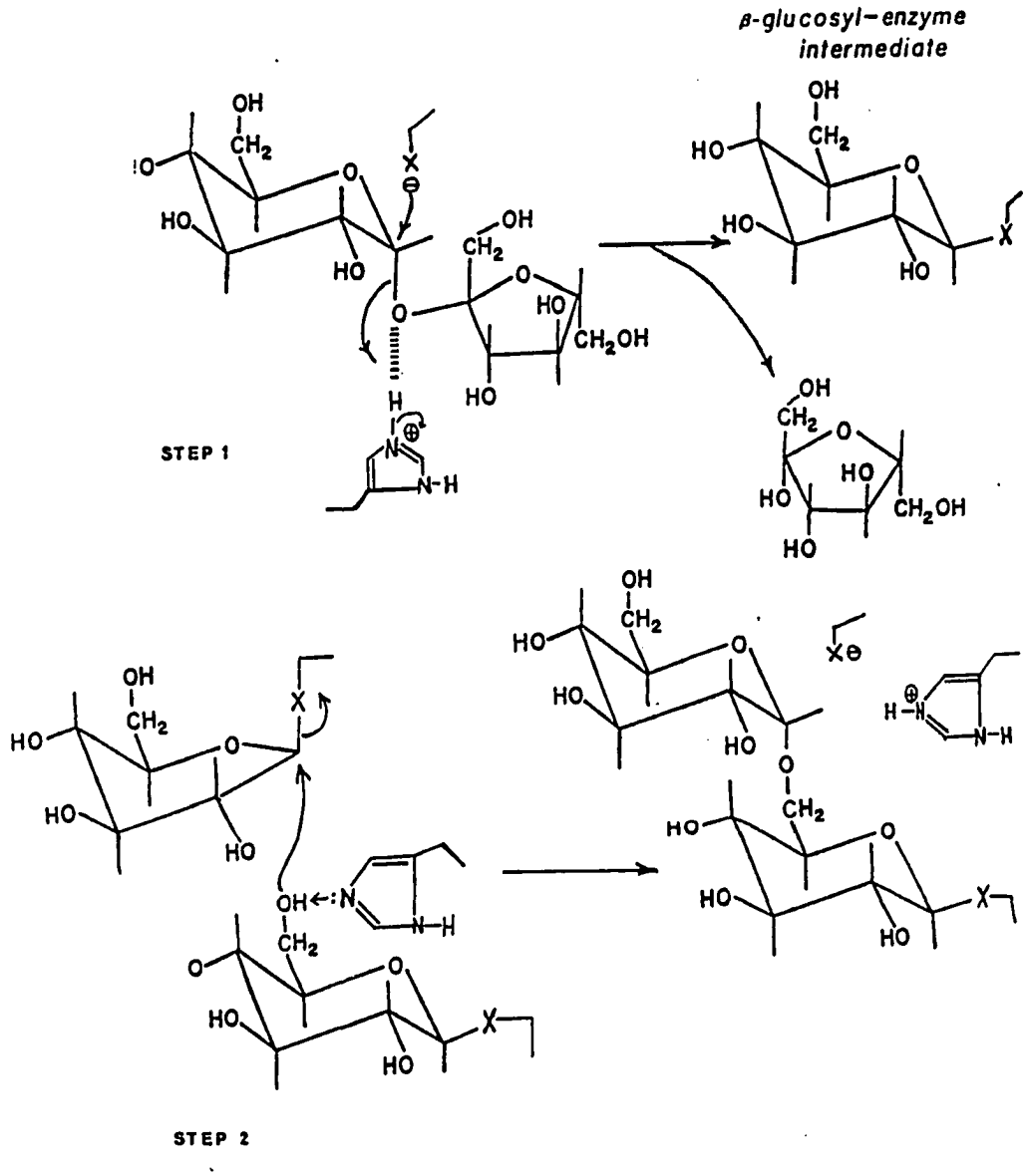

Figure 8. Proposed mechanism of action of the imidazole group at the active site of dextransucrase

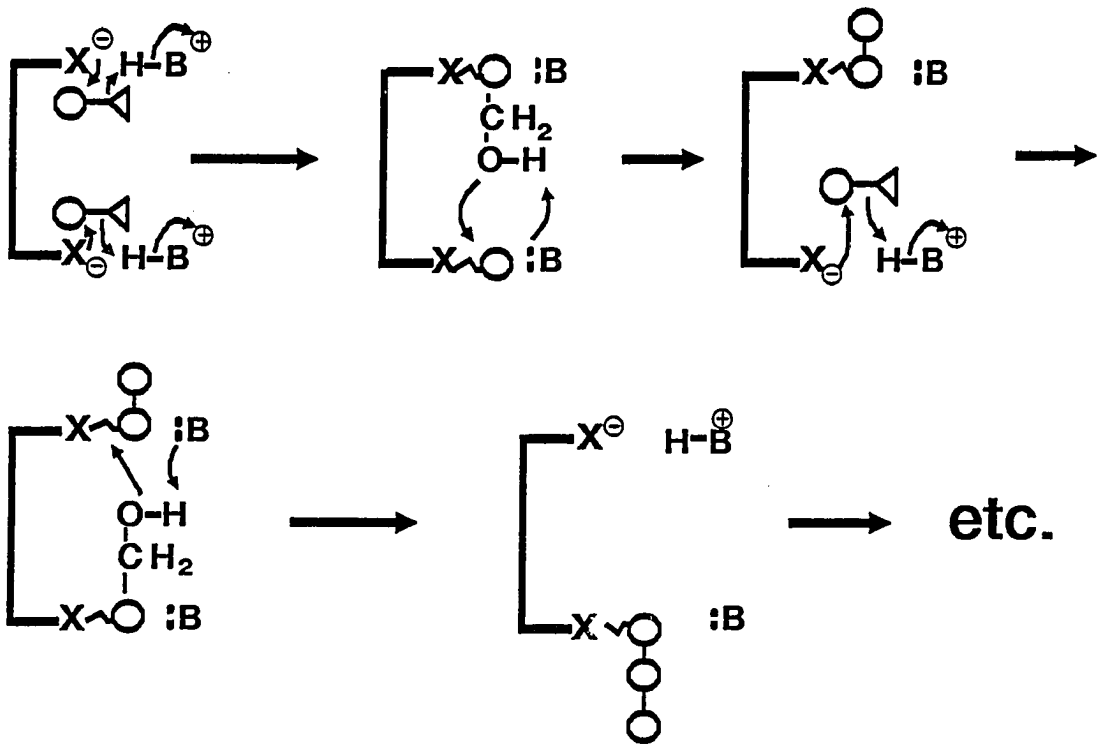

Figure 9. Dextran synthesis of dextransucrase by the insertion mechanism, using two nucleophile (X^-) and two imidazolium groups (HB^+) as catalytic groups at the active site

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SECTION III:

SPECIFICITY OF ACCEPTOR BINDING TO DEXTRANSUCRASE: BINDING AND ACCEPTOR PRODUCTS OF α-METHYL-D-GLUCOSIDE ANALOGUES MODIFIED AT C-2, C-3, AND C-4

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ABSTRACT

The specificity of acceptor binding to the active site of dextransucrase was studied by using α-methyl-D-glucopyranoside analogues modified at C-2, C-3 and C-4 positions by (a) inversion of the hydroxyl group and (b) replacement of the hydroxyl group with hydrogen. 2-Deoxy-α-methyl-D-glucopyranoside was synthesized from 2-deoxyglucose; 3- and 4-deoxy-α-methyl-D-glucopyranosides were synthesized from α-methyl-D-glucopyranoside; and α-methyl-D-allopyranoside was synthesized from D-glucose. The analogues were incubated with [^{14}C]sucrose and dextransucrase, and the products were separated by TLC and quantitated by liquid scintillation spectrometry. Structures of the acceptor products were determined by methylation analyses and optical rotation. The relative effectiveness of the acceptor-analogues in decreasing order were 2-deoxy, 2-inverted, 3-deoxy, 3-inverted, 4-inverted and 4-deoxy. The enzyme transfers D-glucopyranose to C-6 hydroxyl of analogues modified at C-2 and C-3, to C-4 hydroxyl of 4-inverted, and to C-3 hydroxyl of 4-deoxy analogues of α-methyl-D-glucopyranoside. The data indicated that the hydroxyl group at C-2 is not as important for acceptor binding as the hydroxyl groups at C-3 and C-4. The hydroxyl group at C-4 is particularly important as it determines the binding orientation of the α-methyl-D-glucopyranoside ring.

INTRODUCTION

Dextransucrase of Leuconostoc mesenteroides B-512FM polymerizes the D-glucopyranosyl group of sucrose to produce α-(1→6) linked dextran with 5% α-(1→3) branch linkages. When carbohydrates other than sucrose are present in the reaction digests, D-glucopyranosyl residues are transferred to those carbohydrates, which are called acceptors[1-4]. Acceptors range from monosaccharides to polysaccharides. Some of the acceptor products are also acceptors, and in these cases, a series of homologous acceptor products result. One such example is α-methyl-D-glucopyranoside, which gives a homologous series of α-methyl-isomaltodextrins in which each member of the series acts as an acceptor to form the next acceptor product[1,3]. For other acceptors, the first acceptor product is a poor acceptor or a non-acceptor. One such example is D-fructopyranose, which gives leucrose as an acceptor product[2].

Many carbohydrates act as acceptors. A total of seventeen different acceptors has been quantitatively studied[5]. Among the monosaccharides and their derivatives, α-methyl-D-glucopyranoside is the most effective acceptor for dextransucrase. This effectiveness implies that α-methyl-D-glucopyranoside binds to dextransucrase in a favorable manner to accept a D-glucopyranosyl group at its C-6 hydroxyl group. D-Glucopyranose is also an acceptor and gives a homologous series of isomaltodextrins as products. It is, however, only 33% as effective as α-methyl-D-glucopyranoside[5]. This result may arise because D-glucopyranose is composed of an equilibrium mixture of α-and ß-anomers in a ratio of 1:2, and ß-methyl-D- glucopyranoside is only 20% as effective as the α-methyl anomer[5]. By contrast, D-galactose and D-mannose, due to their structural and conformational differences

from D-glucose, are very poor acceptors. They give the unusual nonreducing disaccharides, α-D-glucopyranosyl-ß-D-galactofuranoside and α-D-glucopyranosyl-ß-D-mannopyranoside[5-7].

The location of the acceptor binding site is not known. Further, little is known about the relationship between the acceptor structure and the binding specificity except for the differences observed for the α- and ß-D-glucopyranosyl anomers mentioned above. We, therefore, have studied the specificity of the acceptor binding site by using α-methyl-D-glucopyranoside analogues. We choose to modify α-methyl-D-glucopyranoside because it is the most effective acceptor among the simple monosaccharides. We have studied two types of modifications of the hydroxyl groups at C-2, C-3, and C-4: (a) inversion and (b) replacement with hydrogen. These analogues were chosen to assess the effects of changes of configuration or omission of a single hydroxyl group at specific positions on the sugar ring. The relative efficiencies and the structures of the products of these analogues acting as acceptors have been determined. α-Methyl-D-xylopyranoside and α-and ß-methyl-L-idopyranosides were also examined. This study gives a better understanding of the specificity of acceptor binding to B-512FM dextransucrase.

MATERIALS AND METHODS

Materials

Carbohydrates and reagents

[U-^{14}C]Sucrose was obtained from New England Nuclear (Boston, MA). α-Methyl-D-glucopyranoside was purchased from Eastman Kodak Company (Rochester, NY), and was recrystallized from hot ethanol before use. α-Methyl-D-mannopyranoside, α-methyl-D-galactopyranoside and L-idose were purchased from Sigma Chemical Co. (St. Louis, MO). α-Methyl-D-xylopyranoside was purchased form Aldrich Chemical Co. (Milwaukee, WI). 2-Deoxy-D-glucose was purchased from PCR Research Chemicals, Inc. (Gainesville, FL). Baker's yeast (Red star brand) was obtained from a local food store. Silica gel for column chromatography (40-140 mesh) was purchased from J.T. Baker Inc. (Jackson, TN). Whatman K5F TLC plates were purchased from Whatman Chemical Separation Inc. (Clifton, NJ). Analtech HPTLC plates were purchased from Analtech (Newark, DE). All other chemicals were of reagent grade and commercially available.

Enzyme

L mesenteroides B-512FM[6] dextransucrase (70 IU/mg) was purified through the stage of DEAE-cellulose chromatography as reported previously[6]. Enzyme activity was determined by a radioactive assay using [^{14}C]sucrose[6], and is given in International Unit (IU), that is, in μmol of D-glucose incorporated into dextran per min at pH 5.2 and 25°.

Methods

2-Deoxy-α-methyl-D-glucopyranoside

Dry HCl-methanol solution was prepared by passing dry HCl gas through dry methanol for 10 minutes[8]. 2-Deoxy-D-glucose

(2 g) was dissolved in 200 mL dry HCl-methanol and allowed to react for 2 hours at 20°C. The HCl was neutralized by adding sodium bicarbonate. The methanol was evaporated, and the mixture was chromatographed on a silica gel column (3.5 x 60 cm), which was irrigated with 2% water in 98% acetonitrile. The eluant containing 2-deoxy-α-methyl-D-glucopyranoside was evaporated, and the residue was dissolved in ethylacetate-methanol (30:1 v/v). The solution was warmed on a steambath, and hexane was added slowly until the solution become cloudy. 2-Deoxy-α-methyl-glucopyranoside crystallized overnight. The yield was 1.4 grams (65%). The proton decoupled ^{13}C NMR spectrum had seven peaks: 98.6, 72.6, 71.7, 68.8, 61.8, 54.5, and 37.3 ppm relative to deuterated acetone. A triplet at 37.3 ppm in the proton coupled ^{13}C NMR spectrum showed that the hydroxyl group at C-2 had been replaced by a hydrogen. The $[\alpha]^{20}_D$ in water was + 128.5°.

3-Deoxy-α-methyl-D-glucopyranoside

4,6-Benzylidene-α-methyl-D-glucopyranoside was prepared from α-methyl-D-glucopyranoside by the method of Richtmyer[9]. 3-deoxy-4,6-benzylidene-α-methyl-D-glucopyranoside was prepared by the method of Barton and McCombie[10]. 3-Deoxy-α-methyl-D-glucopyranoside was prepared by dissolving the benzylidene derivative in 50% acetic acid, warming for 10 minutes at 60°C, and then evaporating the solvent to give the final product. The proton decoupled ^{13}C NMR spectrum had seven peaks: 99.9, 74.1, 68.2, 66.0, 62.5, 55.3, and 36.7 ppm relative to deuterated methanol. A triplet at 36.7 ppm in proton coupled ^{13}C NMR spectrum showed that the hydroxyl group at C-3 had been replaced by a hydrogen.

4-Chloro-α-methyl-D-galactopyranoside

4,6-Benzylidene-α-methyl-D-glucopyranoside (60 g) was treated with benzoyl chloride (70 g) in dry pyridine (500 mL) for 1 hour at 0°C, and kept for 10 hours at 20°C. The solution was then poured slowly into ice-water slush (2 L) containing sodium bicarbonate (90 g). The resulting white foam was filtered, washed with water, and dissolved in chloroform. The solution was washed three times with water, and dried over sodium sulfate[11]. The solvent was evaporated to give 4,6-benzylidene-2,3-dibenzoyl-α-methyl-D-glucopyranoside (86 g, 87%). The benzylidene group was removed in 50% acetic acid as described above to give a syrup of 2,3-dibenzoyl-α-methyl-D-glucopyranoside. The syrup was treated with trityl chloride (60 g) in dry pyridine (500 mL) for 12 hours at 20°C until all of the starting material was converted as shown by TLC[12]. The solution was then treated with sulfuryl chloride (30 g) for 1 hour at 0°C and kept overnight at 20°C. TLC showed that one product was formed. The solvent was evaporated and the residue was then dissolved in methylene chloride, which was washed with saturated sodium bicarbonate solution and water. The methylene chloride was evaporated to give 2,3-dibenzoyl-4-chloro-6-trityl-α-methyl-D-galactopyranoside as a syrup. The trityl group was removed by the method of Baker[12] to give 2,3-dibenzoyl-4-chloro-α-methyl-galactopyranoside as a syrup. Benzoyl groups were removed by treating this syrup with sodium methoxide (0.2 g) in methanol (500 mL) at 40°C for 20 minutes. The methanol solution was concentrated to a syrup which was dissolved in 300 mL of water. The water solution was washed with chloroform, evaporated to a syrup, from which 16 g of crystals of 4-chloro-α-methyl-D-galactopyranoside formed overnight. Total yield was 35%. The proton decoupled ^{13}C NMR spectrum had seven peaks: 101.7, 71.1,

70.2, 69.9, 64.8, 62.9, and 55.8 ppm relative to deuterated methanol.

4-Deoxy-α-methyl-D-glucopyranoside

4-Chloro-α-methyl-D-galactopyranoside (5 g) was refluxed in absolute ethanol (500 mL) containing tributyltin hydride (20 g) and α,α'-azo-isobutyronitrile (5 mg) under nitrogen for 48 hours[13]. The solution was evaporated to a syrup, which was adsorbed on silica gel, and placed onto a silica gel column (3.5 x 60 cm), which was irrigated first with hexane (1 L), and then with acetonitrile (2 L). The acetonitrile eluant was evaporated to a syrup, from which 4-deoxy-α-methyl-D-glucopyranoside crystallized overnight. Yield was 2.8 g (67%). The proton decoupled ^{13}C NMR spectrum had seven peaks: 101.8, 75.5, 69.9, 68.8, 65.9, 55.5 and 36.5 ppm relative to deuterated methanol. A triplet at 36.5 ppm in the proton coupled ^{13}C NMR spectrum showed that the hydroxyl group at C-4 had been replaced by a hydrogen.

α-Methyl-allopyranoside

D-allose was prepared from D-glucose by the method of Stevens[14]. α-Methyl-D-allopyranoside was prepared as described by Evans and Angyal[15]. The proton decoupled ^{13}C NMR spectrum had seven peaks: 101.4, 73.3, 69.4, 68.8, 62.7, 65.0 and 55.0 ppm relative to deuterated methanol.

Acceptor-reaction digests

Dextransucrase acceptor-reaction digests (30 μL) contained 50 mM [U-^{14}C]sucrose (3 μCi), 50 mM acceptor sugar, 20 mM pyridine-acetate buffer (pH 5.3), 2 mM calcium chloride, 0.01% sodium azide, and 40 mIU of dextransucrase. The reaction was conducted for 5 hours at 25°C, at which point all of the sucrose had been consumed. Aliquots (2 μL) were

spotted onto a Whatman K5F TLC plate (20 x 20 cm) and chromatographed using three ascents of nitromethane-water-1-propanol (2:3:5 v/v/v). The products were located by autoradiography. The individual labeled compounds on the plate were quantitated by scraping the silica gel from the plate and counting it by heterogeneous liquid scintillation spectrometry using a toluene cocktail.

Preparations of acceptor products

Dextransucrase acceptor digests (200 mL) contained 80 mM sucrose, 80 mM acceptor sugar, 20 mM pyridine-acetate buffer (pH 5.3), 2 mM calcium chloride, 0.01% sodium azide, and 20 IU of dextransucrase. The reactions were conducted for 12 hours at 25°C. Dextran in the digest was precipitated by adding ethanol (400 mL). The supernatant was evaporated, the residue was dissolved in water (100 mL), treated with Baker's yeast (50 mg) for 12 hours at 37°C to remove all of the D-fructose. The acceptor product was then purified by chromatography on a silica gel column that was irrigated with 95:5 (v/v) acetonitrile-water.

Methylation analysis

Methylations were carried out on 2-4 mg samples previously taken up in water and freeze-dried in vials sealed with split rubber septa. Dry DMSO (2 mL) was injected into vials under vacuum. When the sample dissolved, 0.2 mL Hakomori reagent was injected, and after an hour 0.2 mL of methyl iodide was added[16]. Hydrolysis and acetolysis were performed by the method of Stellner et al[17]. Hydrolysis and acetolysis were limited to 2 hours for 2-deoxy-α-methyl-D-glucopyranoside acceptor product in an effort to save some recognizable fragment of the acid-labile 2-deoxy-D-glucose moiety. Gas chromatography and mass spectroscopy were

conducted as described previously[16] to determine the position of the linkage of D-glucopyranose to the acceptor. Methane was employed as the reactant gas for chemical ionization mass spectra.

RESULTS

All of the analogues of α-methyl-D-glucopyranoside modified at C-2, C-3, and C-4, when incubated with dextransucrase in the presence of sucrose, were shown to be D-glucopyranosyl acceptors giving at least one acceptor product (Fig. 1). The quantitative effects for the formation of acceptor products for the α-methyl-D-glucopyranoside analogues are given in Table I. Each of the analogues, except α-methyl-D-galactopyranoside gave a homologous series of products, which were formed from each of the preceding acceptor products. As with α-methyl-D-glucopyranoside[5], the successive homologues in a series were produced in decreasing amounts. α-Methyl-D-galactopyranoside gave only one acceptor product. α-Methyl-D-xylopyranoside, which has no hydroxymethyl group at C-5, was not an acceptor.

In the synthesis of dextran from sucrose by dextransucrase, leucrose and isomaltulose are always formed as minor acceptor products of the D-fructose, which is released from sucrose by dextransucrase in the polymerization reaction. Totally they accounted for 1.8% of the D-glucose incorporated in the absence of acceptor, and 0.7% in the presence of a good acceptor (see Table I). A small amount of free D-glucose is also produced by the action of water as an acceptor[5,18].

In this study, an equal molar ratio of acceptor to sucrose was used in all of the acceptor digests. α-Methyl-D-glucopyranoside was the most effective acceptor, giving a series of homologous acceptor products up to d.p. 10, which together contained 43% of the D-glucopyranosyl residue of sucrose. Its efficiency was taken as 100% in Table I. The relative efficiencies of the other acceptors were calculated accordingly. Of all the analogues of α-methyl-D-gluco-

Figure 1. Acceptor reactions of B-512FM dextransucrase in the presence of no acceptor (1), of α-methyl-D-glucopyranoside (2), of α-methyl-D-mannopyranoside (3), of 2-deoxy-α-methyl-D-glucopyranoside (4), of α-methyl-D-allopyranoside (5), of 3-deoxy-α-methyl-D-glucopyranoside (6), of α-methyl-D-galactopyranoside (7), and of 4-deoxy-α-methyl-D-glucopyranoside (8). The acceptor reaction digests and the chromatogram were prepared as described in Methods with the exception that cold sucrose was used. A is the acceptor used in each individual acceptor reaction. P1 and P2 are the first and second acceptor products for each individual acceptor reaction

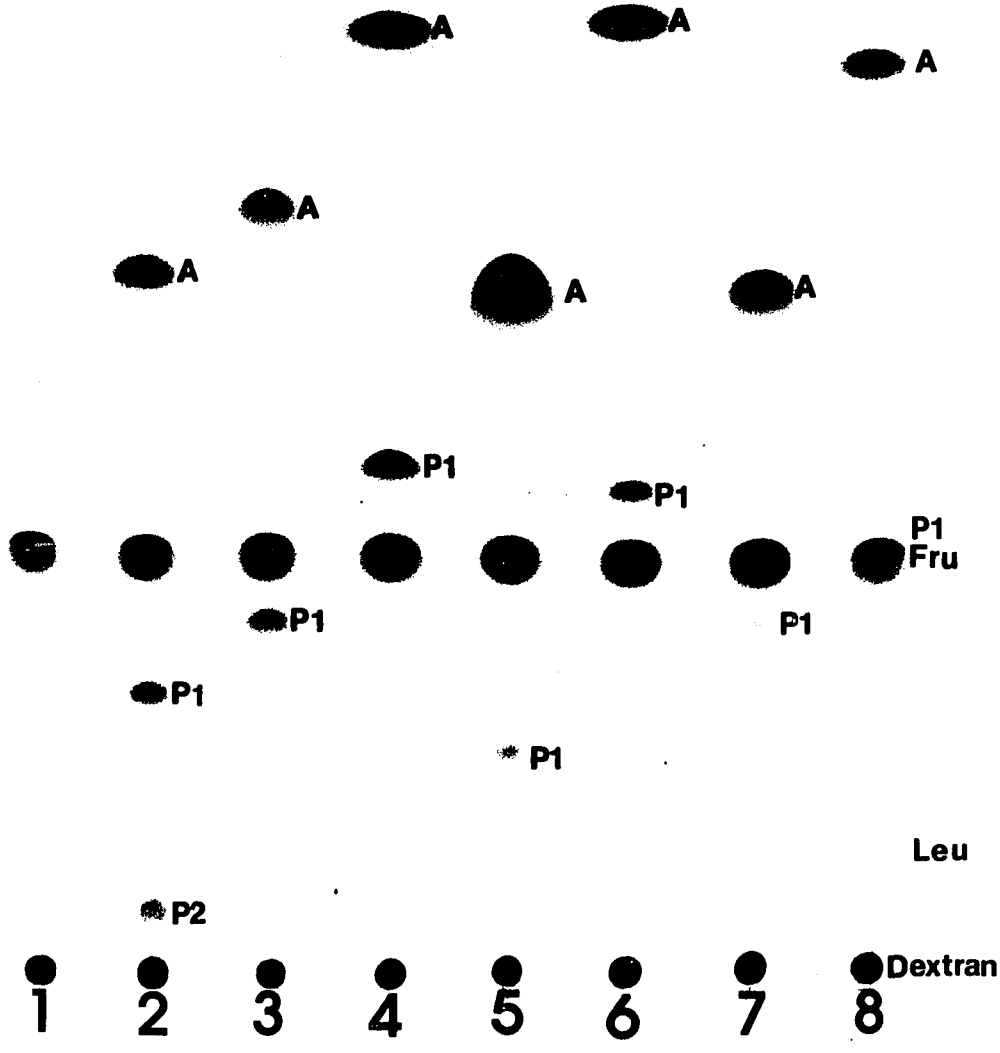
A
A
A
A
A
A
A
P1
P1
P1
P1
P1
P1
P1
Fru
Leu
P2
Dextran
1
2
3
4
5
6
7
8

Table I. Percent of D-glucose from sucrose in products of B-512FM dextransucrase acceptor reactions

Acceptor	Dextran	Leucrose & isomaltulose	Acceptor products			
			Total	dp.2	dp.3	dp.4
α-Me-D-glucopyranoside	55.4	1.20	43.4	14.4	14.7	7.77
α-Me-D-mannopyranoside	80.4	1.31	18.2	16.1	1.03	0.71
2-Deoxy-α-Me-D-glucopyranoside	67.3	0.72	32.0	26.1	2.67	1.81
α-Me-D-allopyranoside	92.5	1.30	6.16	4.98	0.37	0.44
3-Deoxy-α-Me-D-glucopyranoside	81.7	1.43	16.9	12.8	1.85	0.82
α-Me-D-galactopyranoside	94.2	1.76	4.00	4.00	0	0
4-Deoxy-α-Me-D-glucopyranoside	95.5	1.61	2.85	2.15	0.70	0
α-Me-D-xylopyranoside	98.7	1.30	0	0	0	0
No Acceptor	98.2	1.80	0	0	0	0

[a]The relative efficiency of α-methyl-D-glucopyranoside was taken as 100%.

d.p.5	dp.6	dp.7	dp.8	dp.9	dp.10	Relative acceptor efficiency
3.36	1.57	0.90	0.37	0.21	0.11	100[a]
0.30	0.14	0	0	0	0	42.0
0.82	0.33	0.24	0	0	0	73.7
0.37	0	0	0	0	0	14.2
0.73	0.34	0.21	0.13	0	0	39.0
0	0	0	0	0	0	9.22
0	0	0	0	0	0	6.57
0	0	0	0	0	0	0
0	0	0	0	0	0	0

pyranoside, the ones modified at C-2 were the most effective acceptors with a relative efficiency of 74% for 2-deoxy-α-methyl-D-glucopyranoside and 42% for α-methyl-D-mannopyranoside. Each analogue gave a series of acceptor products, up to d.p. 6 for 2-deoxy analogue and d.p. 7 for α-methyl-mannopyranoside. The next most efficient acceptors were analogues modified at C-3 with a relative efficiency of 39% for 3-deoxy-α-methyl-D-glucopyranoside and 14% for α-methyl-D-allopyranoside. The analogues modified at C-4 were comparatively poor acceptors with relative efficiencies of only 6.6% for 4-deoxy-α-methyl-D-glucopyranoside and 9.2% for α-methyl-D-galactopyranoside. The 4-deoxy analogue gave two detectable homologous acceptor products of d.p. 2 and d.p. 3, and α-methyl-D-galactopyranoside gave only one acceptor product (see Table I). 4-Chloro-α-methyl-D-galactopyranoside was not observed to be an acceptor. Other carbohydrates tested for acceptor reactions were α, ß-methyl-L-idopyranosides, which have their hydroxymethyl groups at C-5 inverted, and α-methyl-D-xylopyranoside, which has no hydroxymethyl group at C-5. Neither of these were found to be acceptors.

For each of the acceptors, the first acceptor product was purified, and their structures were determined by methylation analyses. A summary of the results of the methylation analyses are given in Table II. The optical rotations of the products and their structures are summarized in Table III. For α-methyl-D-glucopyranoside and all of its C-2 and C-3 analogues, the D-glucopyranosyl group was attached by an α-(1→6) linkage to the acceptors. For the C-4 analogues, the D-glucopyranosyl group was attached by an α-(1→3) linkage to 4-deoxy-α-methyl-D-glucopyranoside and by an α-(1→4) linkage to α-methyl-D-galactopyranoside.

Table II. Methylation data of the acceptor products

O-Methyl ethers	Mole percentage of methylated sugars from acceptor products[a]					
	1	2	3	4	5	6
2,3,4,6-Me_4-D-Glc	48	66	71	59	58	50
2,3,4-Me_3-D-Man	52					
2-deoxy-4-Me-Hex A[b]		14				
2-deoxy-4-Me-Hex B[b]		20				
2,3,4-Me_3-D-All			29			
3-deoxy-2,4-Me_2-Glc				41		
2,3,6-Me_3-D-Gal					42	
4-deoxy-2,6-Me_2-D-Glc						50

1. product of α-Me-D-mannopyranoside
2. product of 2-deoxy-α-Me-D-glucopyranoside
3. product of α-Me-D-allopyranoside
4. product of 3-deoxy-α-Me-D-glucopyranoside
5. product of α-Me-D-galactopyranoside
6. product of 4-deoxy-α-Me-D-glucopyranoside

[a]Theoretically, each moiety of a permethylated disaccharide should be obtained in 50% mole yield; lower yields reflect decomposition during hydrolysis.

[b]A and B are two isomeric derivatives of 2-deoxy-4-methylhexose generated by loss of a 3-O-methyl during hydrolysis.

Table III. Structures and optical rotations ($[\alpha]^{20}_D$) of acceptor products

Acceptor	Product	$[\alpha]^{20}_D$
α-Me-D-glc-p	6-O-α-D-Glucopyranosyl-α-Me-D-glucopyranoside	+177.4°[a]
α-Me-D-man-p	6-O-α-D-Glucopyranosyl-α-Me-D-mannopyranoside	+118.4°
2-Deoxy-α-Me-D-glc-p	6-O-α-D-Glucopyranosyl-2-deoxy-α-Me-D-glucopyranoside	+138.6°
α-Me-D-all-p	6-O-α-D-Glucopyranosyl-α-Me-D-allopyranoside	+133.9°
3-Deoxy-α-Me-D-glc-p	6-O-α-D-Glucopyranosyl-3-deoxy-α-Me-glucopyranoside	+170.7°
α-Me-D-gal-p	4-O-α-D-Glucopyranosyl-α-Me-D-galactopyranoside	+116.1°
4-Deoxy-α-Me-D-glc-p	3-O-α-D-Glucopyranosyl-4-deoxy-α-Me-D-glucopyranoside	+137.8°

[a]As reported by Jones et al.[1]

DISCUSSION

The acceptor reactions of L. mesenteroides B-512FM dextransucrase have been extensively studied[1-5]. Robyt and Walseth[3] have proposed that acceptors act as nucleophiles in which a hydroxyl group (C_6-OH for D-glucose and maltose, C_5-OH for D-fructopyranose) attacks C-1 of the glucopyranosyl or dextranosyl groups in the enzyme complex. Many carbohydrates can serve as acceptors. A total of 17 acceptors, with acceptor products of known structures, have been quantitatively studied[5]. Very little, however, is known about the structural requirements of the acceptors for dextransucrase. Knowledge of this type is of importance in understanding the interaction of acceptor with enzyme, and should lead to a better understanding of the mechanism of the acceptor reaction. We have investigated acceptor binding specificity of dextransucrase by preparing a series of deoxy and inverted hydroxyl α-methyl-D-glucopyranoside analogues, and examining their relative efficiencies as acceptors and the structures of their acceptor products.

It was found that all of the analogues of α-methyl-D-glucopyranoside, modified at C-2, C-3, and C-4 by inversion of the hydroxyl group or by replacement of the hydroxyl group with hydrogen, gave a series of homologous acceptor products, except α-methyl-D-galactopyranoside for which only one acceptor-product was observed (see Table I). The C-2 analogues were found to be the most efficient, each give rise to a series of homologous acceptor products. This result suggested that a change of the hydroxyl group at C-2 has the least effect on acceptor efficiency, and that this hydroxyl group has a minimal requirement for the binding of α-methyl-D-glucopyranoside to the enzyme. The C_2-OH inverted analogue is 30% less efficient than the 2-deoxy

analogue, which suggests that some steric hindrance might occur when the C_2-OH is inverted. Unlike α-methyl-D-glucopyranoside, however, the first acceptor products (d.p. 2) for the C-2 analogues are poor acceptors themselves. Changes at C-3 and C-4 cause significant alteration in analogue binding to the enzyme. The 3-deoxy analogue is 39% as efficient, compared with 74% for the 2-deoxy analogue; and the C_3-OH inverted analogue is only 14% as efficient as compared with 42% for the C_2-OH inverted analogue. This observation suggests that the C_3-OH on the pyranoside ring is more important in binding to the enzyme acceptor site, and the inversion of the C_3-OH produces steric hindrance for binding. The C-4 analogues were even poorer acceptors, with relative efficiencies of only 6.6% for 4-deoxy and 9.2% for C_4-OH inverted. Furthermore, D-glucopyranosyl groups were attached to 4-deoxy-α-methyl-D-glucopyranoside by an α-(1→3) linkage and to α-methyl-D-galactopyranoside by an α-(1→4) linkage. Consequently, the C_4-OH is not only important for the binding of the acceptor, but it also determines the orientation of the binding of the acceptor ring. When the C_4-OH is inverted, or replaced by a hydrogen, the analogue binds in a different orientation so that the C_3-OH for the 4-deoxy or the C_4-OH for the C_4-OH inverted analogue accepts the D-glucopyranosyl group to form the acceptor product. The C_4-OH inverted analogue is apparently a better acceptor than the 4-deoxy analogue as the C_4-OH on the inverted analogue is closer to the C_6-OH group than C_3-OH is on the 4-deoxy analogue. The α-methyl-D-xylopyranoside (without a hydroxymethyl group at C-5) and the α- and ß-methyl-L-idopyranosides (with an inverted hydroxymethyl group at C-5) were not acceptors. Either they bind but cannot accept the D-glucopyranosyl group because of the changes at C-5, or they cannot bind. The latter is more

likely as indicated by α-methyl-D-glucopyranoside analogues modified at C-6. Various C-6 modified analogues have been prepared, such as 6-deoxy- and 6-deoxy-6-fluoro-α-methyl-D-glucopyranoside[19]. These analogues were very weak acceptors and were not inhibitors for B-512FM dextransucrase[19].

The active site of B-512FM dextransucrase has been previously studied by use of sucrose analogues[13] and chemical modifications of the enzyme[20]. Little, however, is known about the relationship between sucrose and acceptor binding. 6-Deoxy- and 6-deoxy-6-fluoro-sucroses were both strong competitive inhibitors of B-512FM dextransucrase. 3- and 4-deoxysucroses, however, were very poor noncompetitive inhibitors[13]. These results showed that C_3-OH and C_4-OH are important for the binding of sucrose to the active site, whereas the C_6-OH is not. α-D-Glucopyranosyl fluoride is known to act as a substrate for dextransucrase[21-23]. Recently, 6-deoxy-α-D-glucopyranosyl fluoride was synthesized, and was found to be a competitive inhibitor of sucrose for B-512FM dextransucrase with a Ki of 4 mM[19]. These results suggest that α-D-glucopyranosyl fluoride binds to the enzyme in a similar way as sucrose binds, and the C_6-OH is not important for the binding of either sucrose or glucopyranosyl fluoride. It would appear that α-methyl-D-glucopyranoside binds to the active site differently than does sucrose, or binds to a different site, since 6-deoxy-α-methyl-D-glucopyranoside and 6-deoxy-6-fluoro-α-methyl-D-glucopyranoside do not competitively inhibit dextran synthesis, whereas 6-deoxysucrose and 6-deoxy-6-fluorosucrose are potent inhibitors. The C_6-OH is required for acceptor reaction. Hence, α-methyl-D-xylopyranoside, which has no C_6-OH group, and methyl-L-idopyranoside, which has the C_6-OH group inverted, should not bind to the acceptor site. Mayer[24] has reported that several C-6 modified α-methyl-D-glucopyrano-

side analogues were acceptors for Streptococcus sanguis dextransucrase. Our observations for the interaction of the C-6 modified α-methyl-D-glucopyranoside with L. mesenteroides B-512FM dextransucrase are opposite to those reported by Mayer for S. sanguis dextransucrase.

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SECTION IV:

STRUCTURAL ANALYSIS OF MALTODEXTRIN ACCEPTOR PRODUCTS WITH DEXTRANSUCRASE

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ABSTRACT

The acceptor product of maltose with Leuconostoc mesenteroides B-512FM dextransucrase is panose (6^2-α-D-glucopyranosyl maltose). The structures of acceptor products with other maltodextrins (G3-G8), however, have not yet been determined. These products could contribute to the formation of dental plaque because maltodextrins are present in the mouth from the action of salivary α-amylase on food starch. Therefore, the structures of the acceptor products of maltodextrins with dextransucrase were studied by using the known specificities of porcine pancreatic α-amylase and α-glucosidase, and by methylation analysis. It has been found that dextransucrase transfers a D-glucopyranosyl residue to either the nonreducing end or to the reducing end residue of the maltodextrins G3-G8, forming an α(1→6) linkage. When a D-glucose was transferred to the nonreducing residue, the first product then serves as an acceptor to give the second product, which serves an acceptor to give the third product, etc. When a D-glucose was transferred to the reducing residue, the first product will not serve further as an acceptor to give further products or it serves as a very poor acceptor to give a small amount of the next homologue. The effectiveness of maltodextrins as acceptors decreased as the size of the maltodextrin chain increased.

INTRODUCTION

Dextransucrase from Leuconostoc mesenteroides B-512FM catalyzes the polymerization of glucopyranosyl moiety of sucrose to form dextran, an α(1→6) linked glucan with 5% α(1→3) branch linkages. This enzyme has received wide attention because its dextran has many medical and industrial applications. The low molecular weight dextran is used as a blood plasma substitute, and dextran derivatives, such as dextran sulfate, can be used as a substitute for heparin, and epichlorohydrin cross-linked dextran is the commercial gel-filtration material called Sephadex[1]. Since the B-512FM dextransucrase is secreted into the culture medium with little contaminating enzyme activities, it serves as an excellent model for mechanistic studies of several glucansucrases which are known to be involved in the formation of dental plaque and tooth decay[2,3].

When other carbohydrates are present in the reaction digest in addition to sucrose, L. mesenteroides B-512FM dextransucrase also carries out acceptor reactions, in which glucopyranosyl units are transferred from the enzyme to the carbohydrates to form acceptor products[4-6]. The acceptors range in size from monosaccharides to polysaccharides. Over 30 different carbohydrates are known to act as acceptors[7]. Seventeen of them have been quantitatively studied[8]. Maltose is the most efficient acceptor, followed in order by isomaltose, nigerose, α-methyl-D-glucopyranoside and D-glucose. For some acceptors, their acceptor products also serve as acceptors; in these instances, a series of homologous acceptor products result. One such example is maltose, which yields a series of acceptor products in which isomaltodextrins of varying lengths are attached to the nonreducing glucosyl residue of maltose by an α(1→6) linkage[6].

For other acceptors, only single products are produced. One such example is fructose, from which an unusual disaccharide, leucrose, is formed[6].

While the structures of acceptor products of maltose are known, the structures of the acceptor products with other maltodextrins, such as maltotriose (G3), maltotetraose (G4), etc., have not yet been determined. These acceptor products are important in that maltodextrins would occur in the mouth from the action of salivary α-amylase on food starch. The resulting acceptor products of the subsequent interactions of the maltodextrins with dextransucrase produced by the oral bacteria would be contributing factors in the formation of dental plaque. Knowlege of their structures might be used to design inhibitors or agents that could reduce or prevent dental plaque formation. We have, therefore, studied the structures of acceptor products of maltodextrins, G3 through G8, with dextransucrase. In this study, we have determined these structures by using the known specificities of α-glucosidase and porcine pancreatic α-amylase (PPA), followed by analysis of their products by TLC. We have also determined the number of products formed for each acceptor, their amounts, and the amounts of dextran formed. The relative effectiveness of the maltodextrins (G2-G8) was determined by comparing the percent of D-glucose incorporated into each of the acceptor products.

MATERIALS AND METHODS

Materials

Carbohydrates and reagents

Maltose was purchased from Sigma Chemical Company (St. Louis, MO). Maltodextrins, G3 through G7, were obtained from Nihon Shokuhin Kako Co. of Japan. Maltooctaose was purified by charcoal column chromatography[9]. [U-^{14}C]Sucrose was purchased from New England Nuclear (Boston, MA), Whatman K5F TLC plates were purchased from Whatman Chemical Separation Inc. (Clifton, NJ). All other chemicals were of reagent grade and commercially available.

Enzymes

L. mesenteroides B-512FM dextransucrase (70 IU/mg protein) was purified through the stage of DEAE-cellulose chromatography as previously reported[10]. Porcine pancreatic α-amylase was purchased from Worthington Biochemicals Corporation (Freehold, NJ). Dextranase and α-glucosidase were purchased from Sigma Chemical Company (St. Louis, MO).

Methods

Enzyme assay

Dextransucrase activity was determined by a radiochemical assay using [U-^{14}C]sucrose[10], and is given in international units (IU), that is, in μmol of D-glucose incorporated per minute into dextran at pH 5.2 and 25°C.

Acceptor-reaction digests

Dextransucrase acceptor reaction digests (10 μl) contained 40 mM [U-^{14}C]sucrose (1 μCi), 40 mM acceptor sugar, 20 mM pyridine-acetate buffer (pH 5.2), 2 mM calcium chloride, 0.02% sodium azide, and 8 mIU of dextransucrase. The reac-

tions were conducted at 25°C for 12 hours, at which point TLC indicated that all of the sucrose had been consumed. Aliquots (5 μL) of the reaction digests were spotted onto a Whatman K5F TLC plate (20 X 20 cm) for ascending chromatography at 25°C with (a) ethylacetate-ethanol-water (2:2:1, v/v/v) or (b) nitromethane-water-1-propanol (2:3:5, v/v/v). These two solvent systems are complementary to each other in their separating properties: solvent (a) resolves P1a and P1b, the first two acceptor products of each maltodextrin acceptor reaction in which the D-glucopyranose unit is attached to the O-C-6 of the reducing or the nonreducing glucosyl residues of the maltodextrins, respectively; and solvent (b) resolves the other homologous products (P2, P3, etc) for each of the maltodextrin acceptor reactions. The quantitative analyses of the acceptor reactions were carried out by determining the radioactivity of product by using a Vanguard 2001 TLC autoscanner.

Preparations of acceptor products

[U-^{14}C]Glucose-labeled acceptor products were prepared by using Whatman K5F TLC plate as described above. After the acceptor products were located by autoradiography, each individually labeled product on the TLC plate was collected by scraping the silica gel from the plate and extracting it with water (0.5 mL) twice. After removing the silica gel by centrifugation, the supernatant containing the labeled product was evaporated to dryness by gently blowing air over it.

Acceptor products for methylation analysis were prepared by using paper chromatography. Acceptor reaction digests were prepared similarly as described above except that unlabeled sucrose was used. Aliquots (0.5 mL) of the digests were spotted onto Whatman 3MM paper (23 X 57 cm) for

descending chromatography at 37°C with 1-propanol-water (7:3, v/v). Products were located by silver nitrate stain of a guide strip. Regions of the chromatogram containing the individual compounds were cut out; the products were eluted with water, and freeze-dried.

PPA digestion of acceptor products

The [U-^{14}C]glucose-labeled acceptor products, prepared as above, were dissolved in 20 mM imidazole buffer (10 μL) of pH 6.5 containing 50 mU of PPA. The reactions were conducted at 25°C for 12 hours. The digests were spotted onto a Whatman K5F TLC plate and developed using solvent (a). The products were located by autoradiography.

Methylation analysis

Methylation analyses were carried out on 2- to 4-mg sample as described previously[11].

Nomenclature of branched oligosaccharides

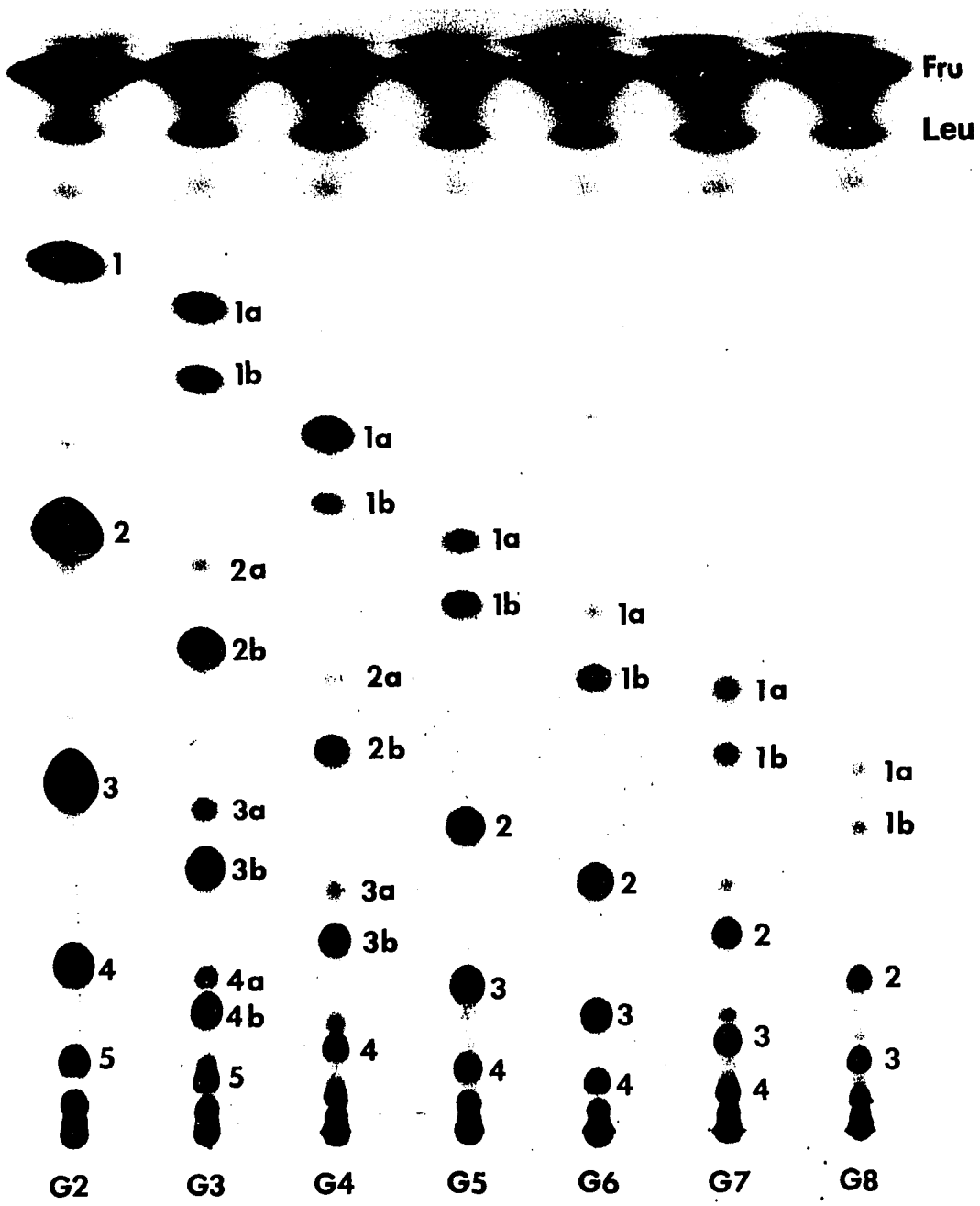
Branched oligosaccharides in this section are named from the reducing end of the main chain by taking the branch as a substitute. The number refers to the hydroxyl group to which the branch is attached, and the superscript refers to the number of the glucosyl residue to which the branch is attached from the reducing end of the main chain. For example, the name for panose is 6^{2}-α-D-glucopyranosyl maltose. The number "6" refers to the hydroxyl group to which the α-D-glucopyranosyl residue is attached, the superscript 2 is the second glucosyl residue of maltose from the reducing end.

RESULTS AND DISCUSSION

All of the maltodextrins (G2-G8) tested, when incubated with dextransucrase in the presence of sucrose, were D-glucopyranosyl acceptors (Fig. 1). The acceptor reaction with maltose (G2) gave panose (6^2-α-D-glucopyranosyl maltose) as the first acceptor product[12], which also served as an acceptor to give 6^2-α-isomaltosyl maltose as the second acceptor product, which went on to give the third, etc. Thus, a homologous series of products were formed, in which isomaltodextrins of varying lengths are attached to the nonreducing glucosyl residue of maltose[8]. The reactions with maltotriose (G3) and maltotetraose (G4) gave two primary products, P1a and P1b (Fig. 1). P1b went on to serve as an acceptor to give a homologous series of acceptor products as P2b and P3b, etc., whereas P1a was a very weak acceptor, the further acceptor products of which (P2a and P3a, etc.) were detectable only by autoradiography. The reaction with maltopentaose (G5) also gave two primary acceptor products (P1a and P1b). P1b went on to give further acceptor products as a homologous series (P2-P6), whereas P1a was not an acceptor. This pattern was repeated for the other maltodextrins (G5-G8) in which two primary acceptor products (P1a and P1b) were formed, but only P1b served further as an acceptor to give a homologous series of acceptor products; while P1a did not further react to give a series of acceptor products.

In this study, an equal molar ratio of acceptor to sucrose was used in all of the maltodextrin-acceptor digests. A quantitative study of their acceptor reactions is summarized in Table I. It was found that maltose was the most effective acceptor, giving a series of seven homologous acceptor products, which together contained 72% of the D-glucosyl

Figure 1. Acceptor reactions of B-512FM dextransucrase with maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7), and maltooctaose (G8). The acceptor reaction digests and autoradiogram were prepared as described in Methods. Products 1a and 1b are the first two primary acceptor products formed by transfer of D-glucose to the acceptor. Products 2a and 2b are the homologous acceptor products that resulted by transfer of D-glucose to 1a and 1b respectively. Product 2 and higher are the homologous products of product 1b when 1a did not further react. A is the acceptor. Fru and Leu are fructose and leucrose respectively


Fru
Leu
1
1a
1b
2
2a
2b
3
3a
3b
4
4a
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5
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2a
2b
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1a
1b
2
3
4
1a
1b
2
3
4
1a
1b
2
3
4
1a
1b
2
3
G2
G3
G4
G5
G6
G7
G8

Table I. Percentage of glucose from sucrose in products of B-512FM dextransucrase acceptor reactions with maltodextrins

Acceptor	Acceptor Products				
	P1a	P1b	P2	P3	P4
G2		14.5	27.4	18.8	7.0
G3[a]	4.1	3.2	6.9	6.5	4.0
G4[a]	4.5	1.5	2.7	2.8	1.9
G5	1.4	1.8	4.1	4.2	2.1
G6	0.4	1.1	2.3	2.2	0.9
G7	0.6	0.8	1.4	1.7	1.1
G8	0.6	0.5	0.9	1.1	0.7

[a]For products P2 through P7, each contains two isomers Pa and Pb which were not resolved resolved.

[b]The relative efficiency of maltose was taken as 100%.

P5	P6	P7	Dextran	Rel. efficiency (%)
2.3	1.1	0.5	28.4	100[b]
2.0	1.2	1.0	71.2	40.3
1.0	1.0		84.6	21.5
0.9	0.4		85.1	20.8
0.4	0.3		92.5	10.5
0.6	0.5		93.3	9.4
0.4	0.3		95.6	6.2

residues of sucrose. Its efficiency was taken as 100% in Table I. The relative efficiencies of the other malto-dextrins (G3-G8) were calculated accordingly. It was shown that the relative efficiencies of maltodextrins as acceptors decreased as the sizes of the acceptors increased. Malto-triose was the next most effective acceptor with a relative efficiency of 40%, having a significant drop of 60% from that of maltose. Maltotriose gave two series of acceptor products; series a and b (Fig. 1). Since P1a was such a poor acceptor, its further acceptor products could not be detected by the TLC autoscanner. The further homologous acceptor products of P1b were, therefore, designated as P2 to P7 in Table I. G4 through G8 were comparatively weak acceptors, with efficiencies of about 20% for G4 and G5, 10% for G6 and G7, and 6% for G8. Each gave a total of seven acceptor products, six of which were of a homologous series formed from P1b and designated P1b-P6.

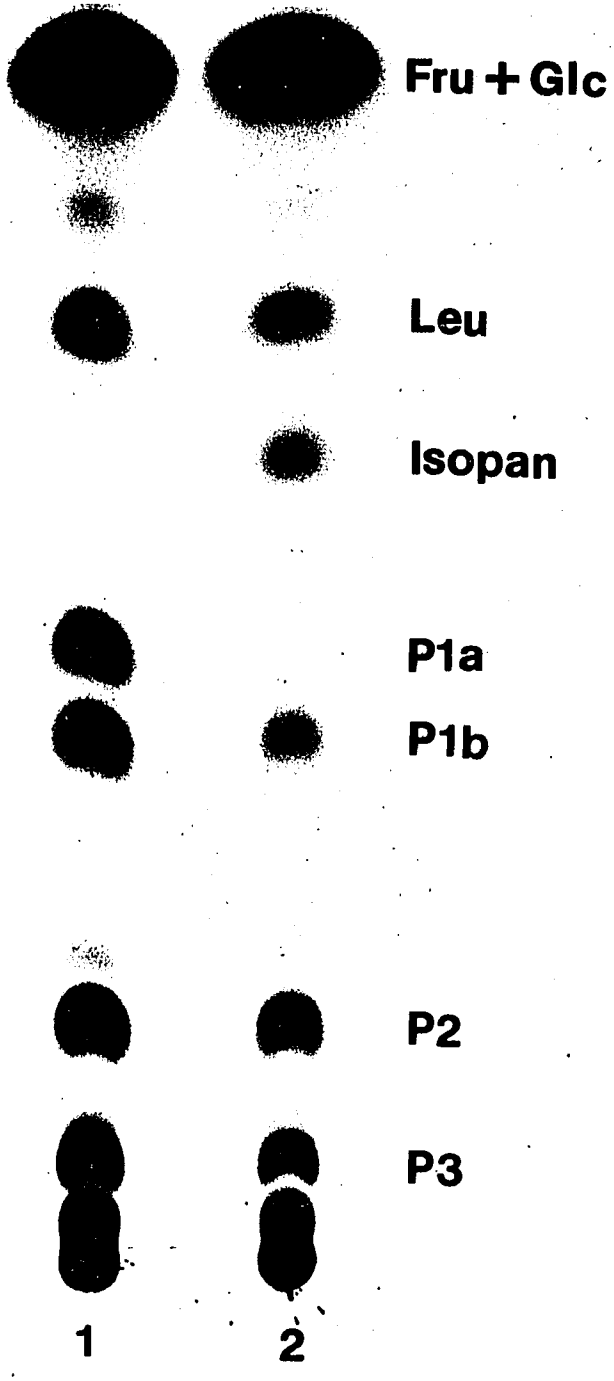
The structures of the acceptor products with maltose has been determined[8] to give a homologous series in which iso-maltodextrins of varying lengths are attached to the C-6 of the nonreducing glucosyl residue of maltose. In the present study, however, it was found that when [U-^{14}C]sucrose of high specific activity of was used, a trace amount of D-glucose from sucrose was transferred to the reducing gluco-syl residue of maltose to give isopanose (see G2R of Fig. 8) in addition to the nonreducing glucosyl residue of maltose to give panose and a series of homologous products. For the other malto-dextrin acceptors (G3-G7), their product struc-tures were determined by using the known specificities of α-glucosidase (for G3) and PPA (for G4 through G7), and by methylation analysis (for G3 and G4).

The acceptor reaction of G3 with dextransucrase gave two primary products P1a and P1b (Fig. 2), for which there are

three possible structures, namely 6^3-, 6^2-, and 6^1-α-D-glucopyranosyl maltotriose. Since α-glucosidase is an exoenzyme that removes glucosyl residues from the nonreducing end, the first compound would be completely resistant to its hydrolysis, while the second would be relatively resistant because of its branch linkage onto the second glucosyl residue, and the third could be hydrolyzed by removing one glucose from the nonreducing end giving isopanose. When the G3 reaction digest was treated with α-glucosidase, only P1a was completely hydrolyzed to isopanose (Fig. 2); whereas P1b, P2 and P3 were left intact. This showed that P1a is the third proposed structure, namely, 6^1-α-D-glucopyranosyl maltotriose, and P1b is the first proposed structure, namely, 6^3-α-D-glucosyl maltotriose. The structures of P2 and P3 were determined by treating them with dextranase, which converted them to P1b, indicating that they were maltotriose units to which isomaltose and isomaltotriose were attached to the nonreducing glucosyl residue by an α(1→6) linkage. P2 and P3 are, therefore, homologous acceptor products of P1b, namely, 6^3-α-isomaltosyl maltotriose (P2b) and 6^3-isomaltotriosyl maltotriose (P3b). P1a was such a weak acceptor that its acceptor products were not visible on the TLC plate. For each of the above products, P1a through P2, milligram quantities were prepared by paper chromatography, and methylation GC-Mass spectrometric analyses were performed. The methylation data agreed with the above structures of the acceptor products. Since 2,3-di-O-methyl glucose was not detected by methylation analysis, the possibility of the second proposed structure, in which D-glucose was attached to the second glucosyl residue of G3, was eliminated.

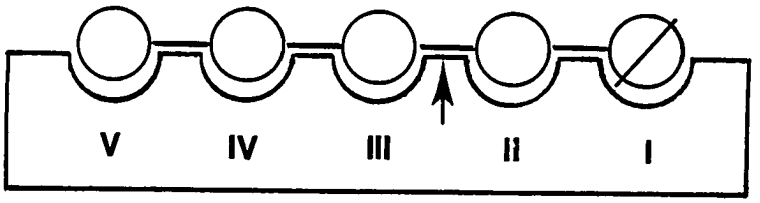
For each of the acceptor reactions of G4 through G7, products P1a through P3 were studied by using the known

Figure 2. Acceptor reactions of B-512FM dextransucrase with G3 (1) and treatment of G3 reaction digest with α-glucosidase (2). The reaction digest, its treatment with α-glucosidase and the autoradiogram were prepared as described in Methods. P1a and P1b are the first two primary products, P2 and P3 are homologous acceptor products of P1b. Fru, Glc, Leu and Isopan are fructose, glucose, leucrose and isopanose respectively


Fru + Glc
Leu
Isopan
P1a
P1b
P2
P3
1
2

specificity of PPA[13]. PPA hydrolyzes amylose or amylopectin to give maltose, maltotriose and maltotetraose as products. A study of the types of labeled products released from reducing-end labeled maltotriose to maltooctaose defined the bond specificity of PPA[13]. It was shown that only maltotriose and maltotetraose give D-glucose as a product. Neither of them are good substrates. The first substrate to undergo hydrolysis at an appreciable rate is maltopentaose, which is exclusively cleaved at bond two[13]. From this investigation, it was postulated that PPA has five D-glucose subsites, I to V, and the catalytic groups are located between the second and the third subsite from the reducing-end subsite (Fig. 3). When acting on amylopectin, PPA can catalyze hydrolysis on both sides of an $\alpha(1\rightarrow6)$ branch linkage. When hydrolyzing from the reducing side of the branch linkage, PPA hydrolyzes only up to three glucosyl residues from the branch linkage[14,15].

For the G4 acceptor reactions, products P1a through P3 were isolated as described in Methods, and were incubated with PPA (Fig. 4). It was found that P1a was relatively resistant to PPA hydrolysis, and only a small amount of it was converted to 6^1-α-D-glucosyl maltotriose (as identified by P1a of the G3 acceptor reactions) as a very minor product and to 6^1-α-D-glucosyl maltose (isopanose) as a major product. 6^1-α-D-glucosyl maltotriose could be formed by removing one glucose from the nonreducing or the reducing end, while isopanose could be formed by removing two glucoses from the nonreducing end or one glucose from each end. Based on the fact that PPA can hydrolyze from the reducing end of a branched maltodextrin only up to three glucosyl residues from the $\alpha(1\rightarrow6)$ branch linkage, P1a of the G4 reactions must be



PPA Active Site

Figure 3. Productive complex of PPA with maltopentaose. The enzyme is shown to have five subsites I to V, with catalytic groups between subsites II and III. From Robyt and French[13]

hydrolyzed from the nonreducing end by removing one glucose to give 6^1-α-D-glucosyl maltotriose and by removing two glucoses to give isopanose. Therefore, the structure of P1a of G4 reactions is 6^1-α-D-glucosyl maltotetraose. This structure is in accord with its relative resistance to PPA hydrolysis and the five subsite model of PPA (Fig. 3). Since G5 and higher maltodextrins are good substrates, while G4 is a somewhat poor substrate and G3 is markedly so[12], a favorable binding of the substrate is achieved only when the five subsites are all occupied by glucosyl residues of the maltodextrins. Therefore, one would expect that P1a of the G4 reactions would be a very poor substrate for PPA because of its branch linkage, and there are the two possible ways of binding to the active site; all four glucosyl residues of G4 portion of P1a bind to subsites I to IV (Scheme 1 of Fig. 5), or only three glucosyl residues 2 to 4 of G4 portion of P1a bind to subsites I to III, leaving the branched-reducing glucosyl residue unbound (Scheme 2 of Fig. 5). Because maltotriose is a much poorer substrate for PPA than maltotetraose, it is expected that the former way of binding (Scheme 1 of Fig. 5) is relatively favorable to give isopanose as the major product by removing maltose from the nonreducing end, while the latter way of binding (Scheme 2 of Fig. 5), in which only three glucosyl residues bind to PPA, is very unfavorable to give 6^1-α-D-glucosyl maltotriose as a very minor product by removing one glucose from the nonreducing end. P1b through P3, however, were readily hydrolyzed by PPA to 6^3-α-D-glucosyl maltotriose, 6^3-α-isomaltosyl maltotriose, and 6^3-isomaltotriosyl maltotriose, as were identified by P1b through P3b of the G3 acceptor products. Since PPA can not hydrolyze the nonreducing glucosyl

Figure 4. PPA digestion of G4 the acceptor products P1a (2), P1b (3), P3 (4), and P3 (5) as described in Methods. G4 acceptor reaction digest was used as standards (1)

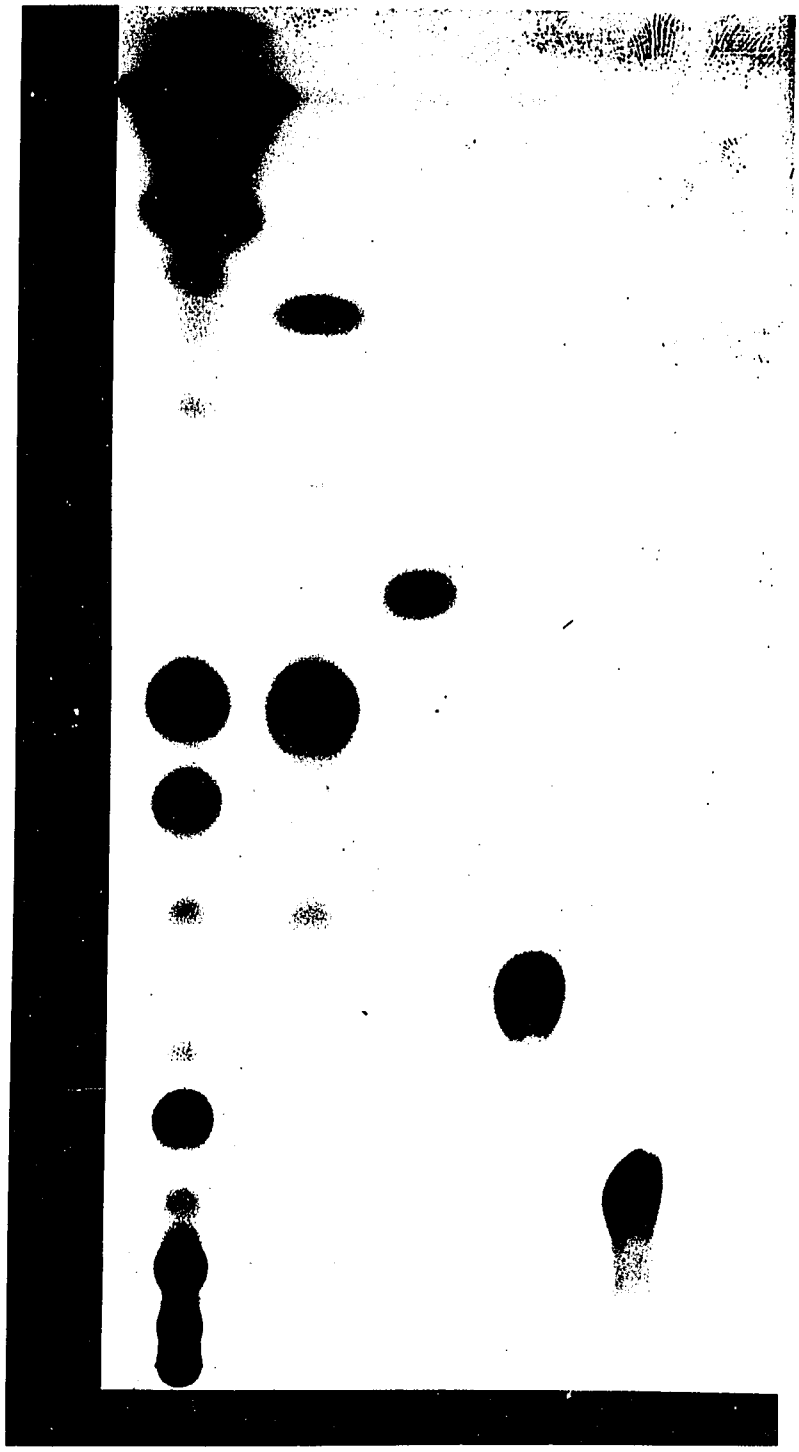


Figure 5. Schematic representations of the action of PPA on G4 through G7 dextransucrase acceptor products

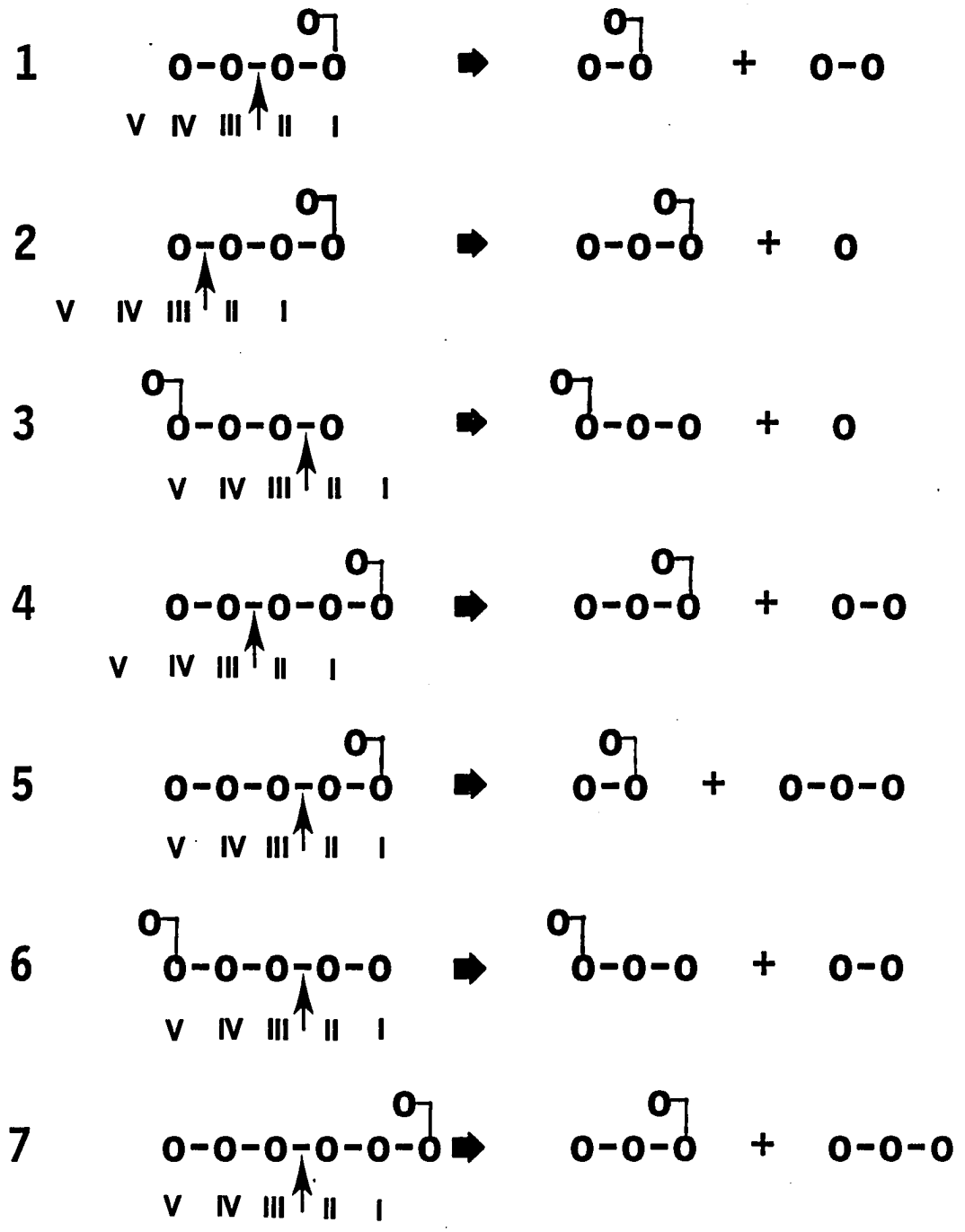
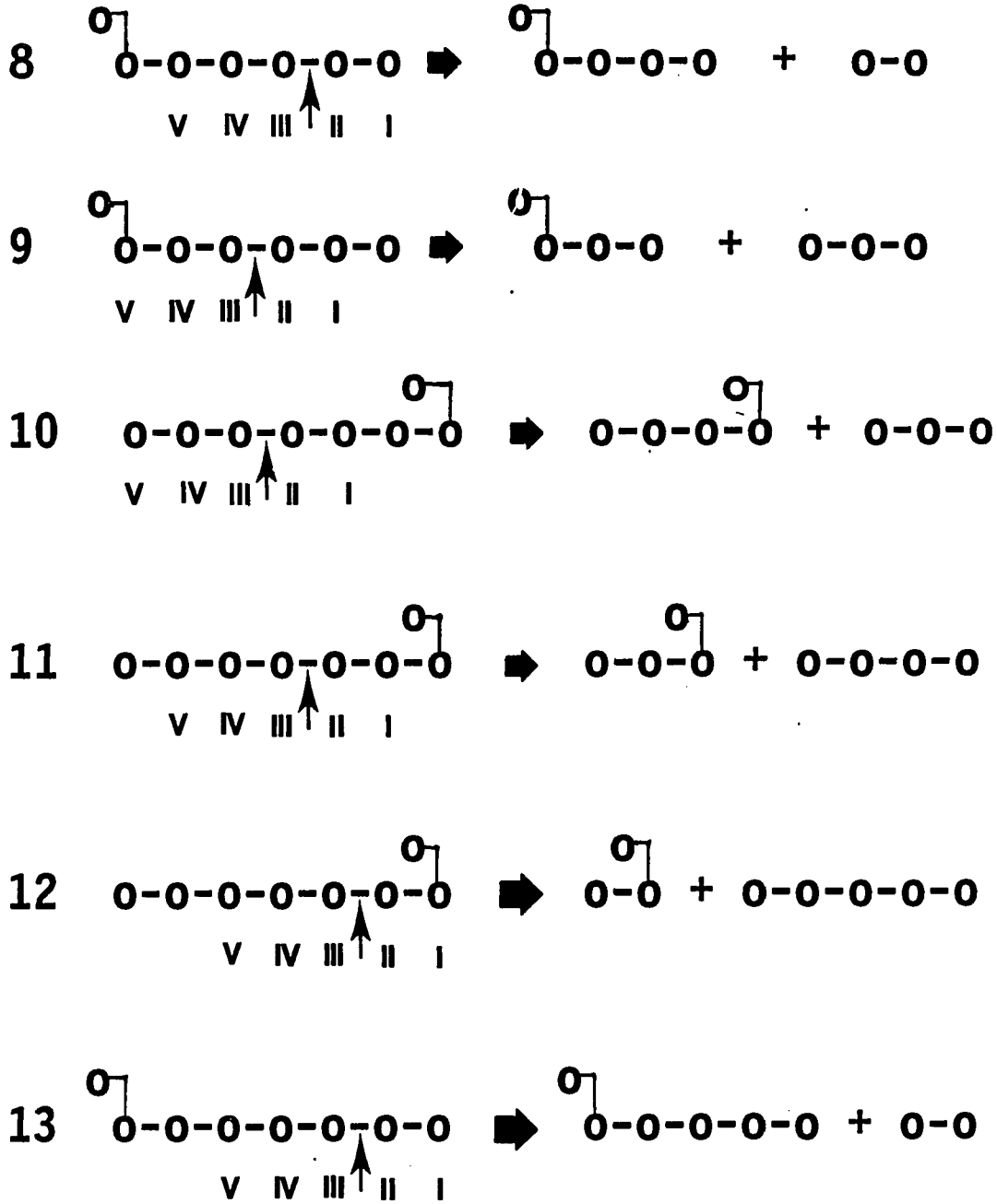

1
O-O-O-O
V IV III II I
O-O + O-O
2
O-O-O-O
V IV III II I
O-O-O + O
3
O-O-O-O
V IV III II I
O-O-O + O
4
O-O-O-O-O
V IV III II I
O-O-O + O-O
5
O-O-O-O-O
V IV III II I
O-O + O-O-O
6
O-O-O-O-O
V IV III II I
O-O-O + O-O
7
O-O-O-O-O-O
V IV III II I
O-O-O + O-O-O

Figure 5 (continued)



residue from 6^3-α-D-glucosyl maltotetraose to give 6^3-α-D-glucosyl maltotriose, products P1b, P2, and P3 of the G4 acceptor reactions must have been cleaved by PPA from the reducing side of the branch linkage by removingone glucose from each of them (Scheme 3 of Fig.4). Therefore, the structures of P1b, P2and P3 of the G4 acceptor products are respectively 6^4-α-D-glucosyl maltotetraose, 6^4-α-isomaltosyl maltotetraose and 6^4-α-isomaltotriosyl maltotetraose. The structures of products P1a through P2 of G4 acceptor reactions were further confirmed by methylation GC-Mass spectrometry anaylsis as described above.

For the G5 acceptor reactions, structures of products P1a through P3 were studied similarly as for the G4 acceptor products (Fig. 6). P1a was slowly hydrolyzed to give 6^1-α-D-glucosyl maltotriose (P1a of the G3 acceptor reactions) as a major product and isopanose as a minor product. Since PPA can only hydrolyze a branched maltodextrins up to three glucosyl residues from the reducing side of the branch point, P1a of G5 acceptor reactions must have been cleaved from the nonreducing end by removing two glucosyl residues to give 6^1-α-D-glucosyl maltotriose and three glucosyl residues to give the isopanose. The structure for P1a of G5 reactions, therefore, is 6^1-α-D-glucosyl maltopentaose. This result is consistent with the five subsite model and the specificity of PPA. Because of the poor tolearnce of subsite I for a branched glucosyl residue, it is expected that PPA would bind more strongly the four glucosyl residues 2 to 5 of G5 portion of P1a to subsites I to IV by leavinng the branched reducing glucosyl residue unbound (Scheme 4 of Fig. 5) than all five glucosyl residues 1 to 5 to subsites I to V (Scheme 5 of Fig. 5). The former way of binding (Scheme 4 of Fig. 5) gave 6^1-α-D-glucosyl maltotriose by removing a maltose from the nonreducing end, while

the latter way of binding (Scheme 5 of Fig. 5) gave isopanose by removing a maltotriose from the nonreducing end. Since subsite V permits the binding of a glucosyl residue with a glucosyl residue attached by an $\alpha(1\rightarrow6)$ linkage, products P1b, P2 and P3 of G5 reactions were readily hydrolyzed to 6^3-α-D-glucosyl maltotriose, 6^3-α-isomaltosyl maltotriose and 6^3-isomaltotriosyl maltotriose by removing two glucosyl residues from the reducing end (Scheme 6 of Fig. 5) as were identified by P1b, P2b and P3b of G3 acceptor reactions. Therefore their structures are respectively 6^5-α-D-glucosyl maltopentaose, 6^5-α-isomaltosyl maltopentaose and 6^5-isomaltotriosyl maltopentaose.

For the G6 acceptor reactions, the structures of the acceptor products P1a through P3 were studied similarly as for the G4 and the G5 acceptor reactions by using the known specificities of PPA (Fig. 7). When incubated with PPA, product P1a was readily cleaved to give exclusively 6^1-α-D-glucosyl maltotriose. For the same reason that PPA can hydrolyze a branched maltodextrin from the reducing end only up to three glucosyl residues from the branch linkage, P1a of G6 reactions apparently was cleaved from the nonreducing end by hydrolyzing three glucosyl residues to give 6^1-α-D-glucosyl maltotriose. One would then expect that the structure of P1a of the G6 acceptor reactions is 6^1-α-D-glucosyl maltohexaose. This structure agreed well with the specificity of PPA, since the only favorable binding of P1a of G6 reactions would use its glucosyl residues 2 to 6 of the G6 portion of P1a to fill the five subsites I to V to give 6^1-α-D-glucosyl maltotriose by removing a maltotriose from the nonreducing end (Scheme 7 of Fig. 5). Products P1b through P3 of G6 acceptor reactions were also readily hydrolyzed by PPA to give the corresponding P1b through P3 of the G3 acceptor reactions by removing a maltose (Scheme 8 of Fig.

Figure 6. PPA digestion of G5 acceptor products P1a (2), P1b (3), P2 (4), and P3 (5) as described in Methods. G3 acceptor reaction digest was used as standards (1)

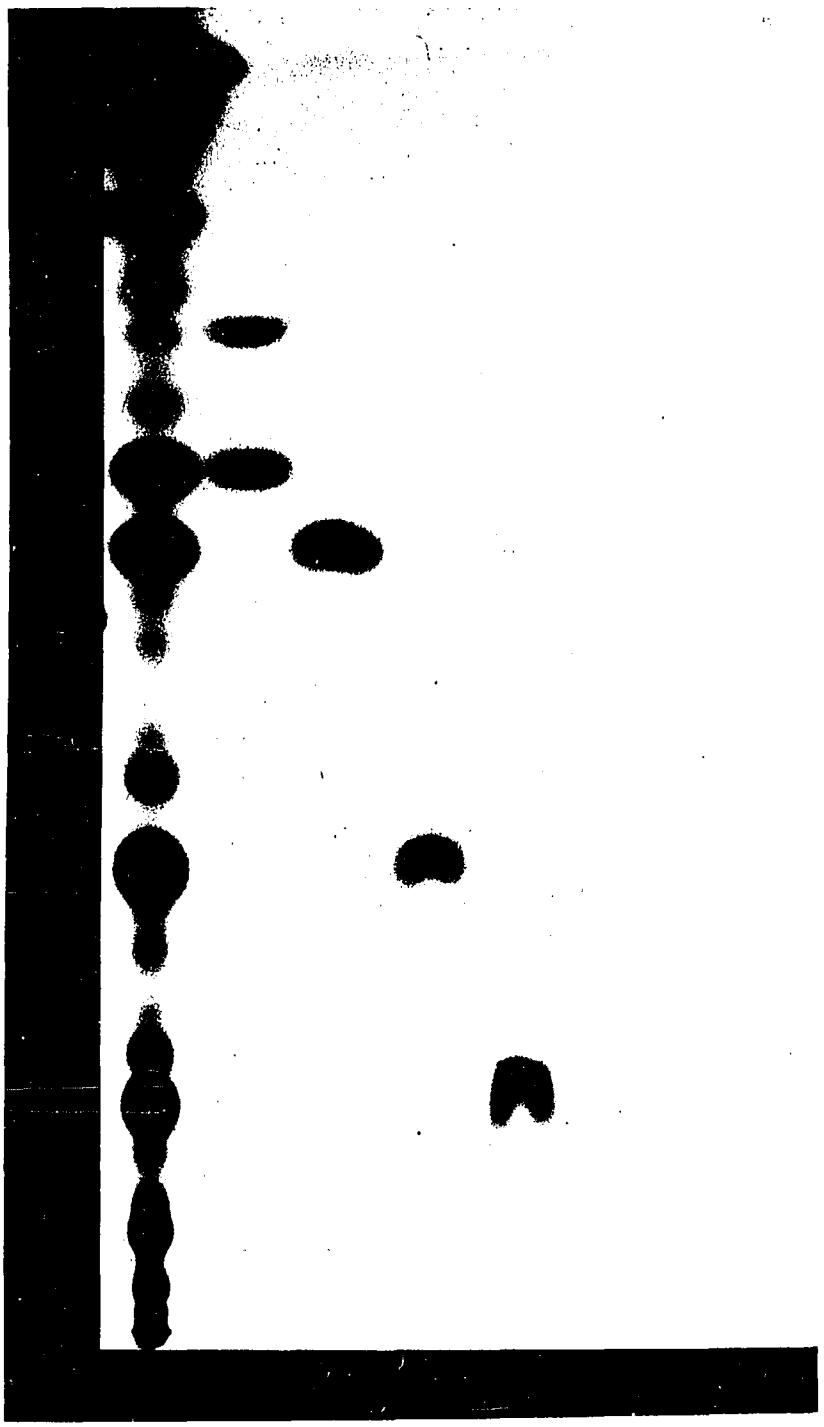
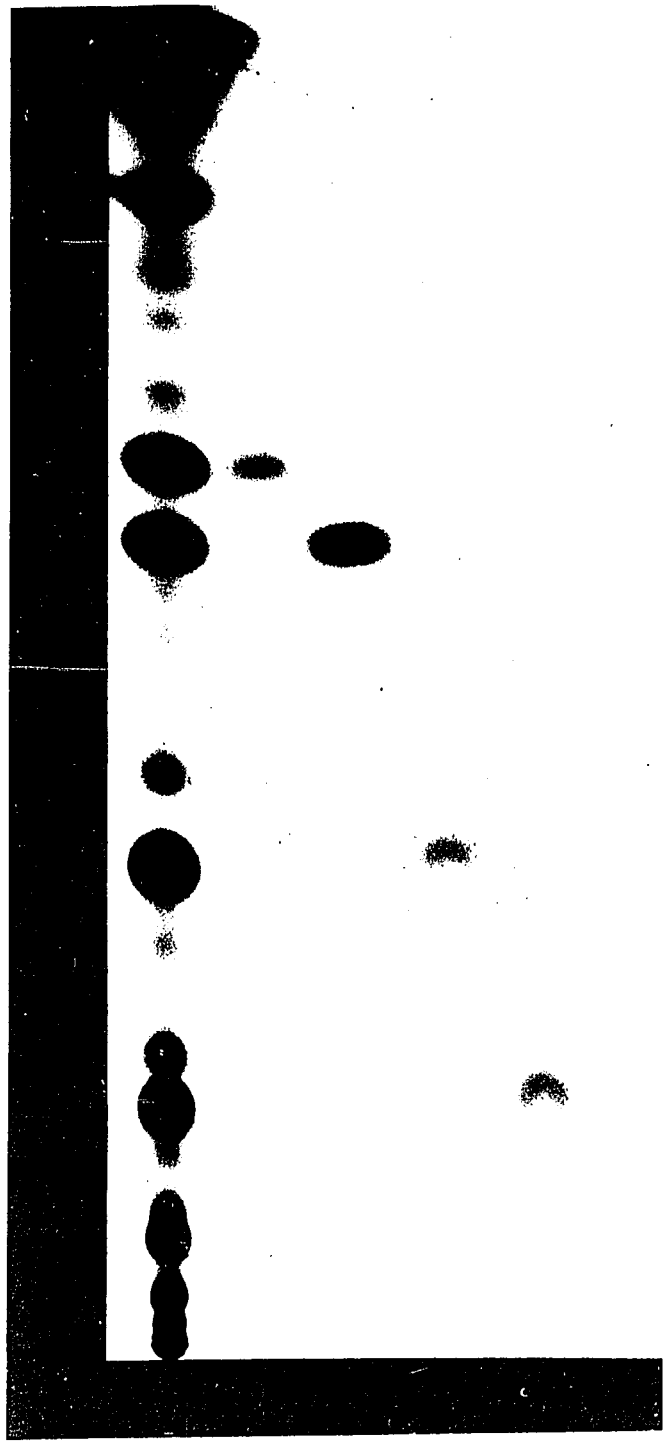


Figure 7. PPA digestion of G6 acceptor products P1a (2), P1b (3), P2 (4), and P3 (5) as described in Methods. G3 acceptor reaction digest was used as standards (1)



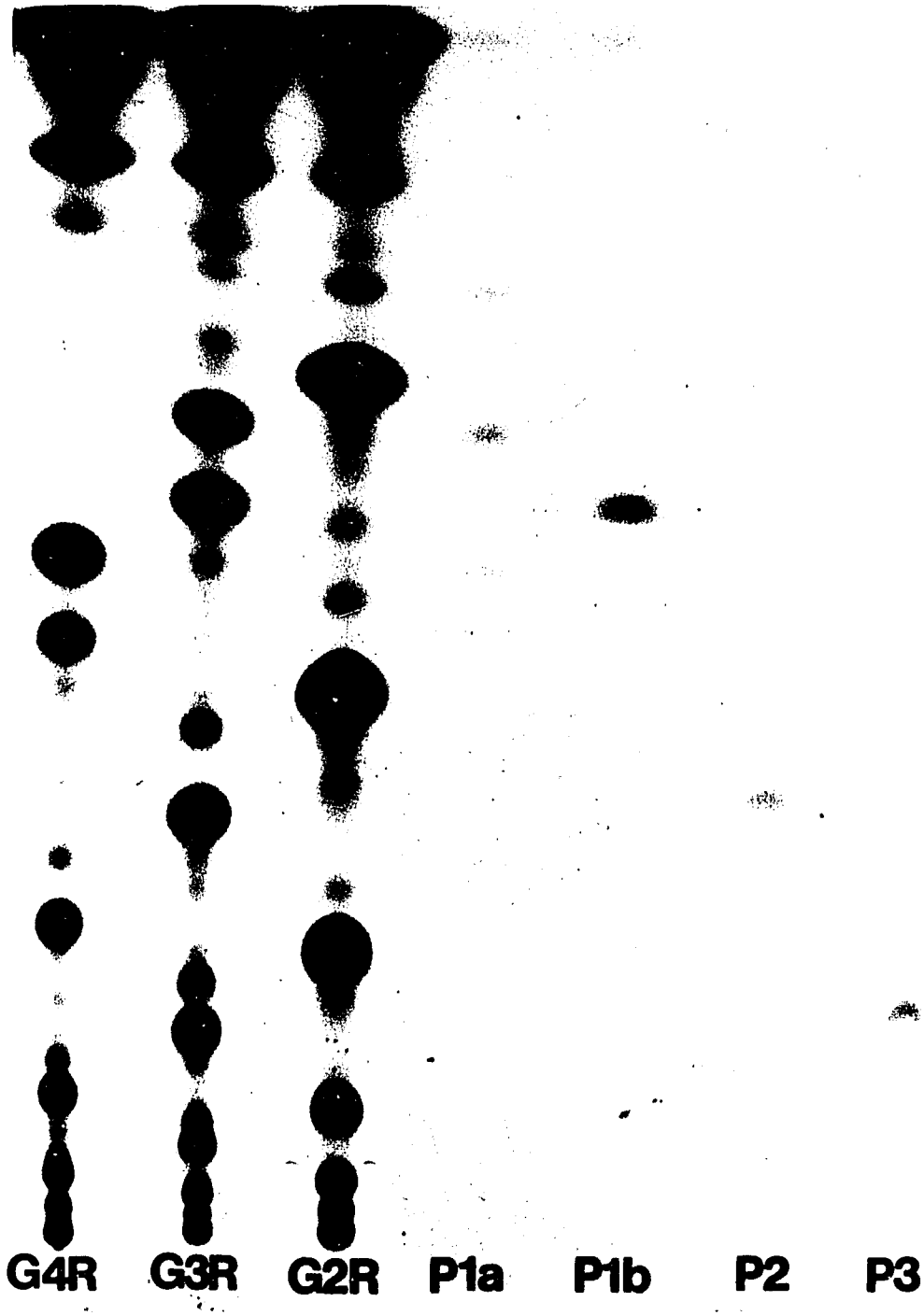
5) and then a glucose (Scheme 3 of Fig. 5), or a maltotriose from the reducing end (Scheme 9 of Fig. 5). Therefore, the structures of P1b through P3 of the G6 acceptor reactions are 6^6-α-D-glucosyl maltohexaose, 6^6-α-isomaltosyl maltohexaose and 6^6-α-isomaltotriosyl maltohexaose.

The acceptor products of the G7 acceptor reactions were also hydrolyzed by PPA (Fig. 8). P1a was hydrolyzed to give 6^1-α-D-glucosyl maltotetraose, 6^1-α-D-glucosyl maltotriose and isopanose as a minor product. Based on the same specificity of PPA, they are formed respectively by removing a maltotriose, a maltotetraose and a maltopentaose from the nonreducing end. The first product, 6^1-α-D-glucosyl maltotetraose, was formed by binding glucosyl residues 3 to 7 of the G7 portion of P1a to the five subsites of PPA (Scheme 10 of Fig. 5). The second product, 6^1-α-D-glucosyl maltotriose, was formed by binding glucosyl residues 2 to 6 of the G7 portion to the five subsites (Scheme 11 of Fig. 5). The third product, isopanose, was formed by binding glucosyl residues 1 to 5 to the five subsites (Scheme 12 of Fig. 5). Since this is an unfavorable binding because of the branch linkage on the reducing glucose, isopanose was produced as a minor product (Fig. 8). Products P1b through P3 were hydrolyzed by removing two maltose units from the reducing end (Schemes 13 and 6 of Fig. 5); their structures are, therefore, 6^7-α-D-glucosyl, 6^7-α-isomaltosyl, and 6^7-α-isomaltotriosyl maltoheptaoses.

As shown in Table I, the relative efficiencies of acceptor reactions with maltodextrins decrease as the size of maltodextrins increase. For the G8 acceptor reaction, only 4% of the glucose from sucrose was incorporated into the acceptor products. Its acceptor products, therefore, were not specifically studied. However, since the G8 acceptor reactions followed the same pattern as G3 through G7 by giving

two primary products P1a and P1b, and one of which went on to give a series of homologous products. It is reasonable to expect the same products for G8 acceptor reactions as for the other maltodextrins, G3 through G7, in which dextransucrase transfers a D-glucosyl residue to the reducing residue of G8 to give P1a (6^1-α-D-glucosyl maltooctaose and to the nonreducing residue to give P1b (6^8-α-D-glucosyl maltooctaose), which went on to give a homologous series of products in which isomaltodextrin chains of varying lengths are attached to the nonreducing end glucose unit of G8.

Figure 8. PPA digestion of G7 acceptor products P1a, P1b, P2 and P3 as described in Methods. G2, G3, and G4 acceptor reaction digest was used as standards


G4R
G3R
G2R
P1a
P1b
P2
P3

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GENERAL CONCLUSION

As stated in the general introduction of the dissertation, the objective of this research was to study the active site and mechanism of dextransucrase, thereby providing the basic information leading to a fundamental understanding of structure and function of enzymes, or more specifically to a clearer picture of the two different reaction mechanisms of dextran polymerization and acceptor reactions and in the future development of anti-caries agents. While the complete goal of understanding the structure and function of dextransucrase is yet to be achieved, the results of this research have provided further groundwork towards this goal.

The work on dextransucrase purification carried the subject a little further than previous studies by producing a homogeneous preparation of dextransucrase with relatively high specific activity, low carbohydrate content and reasonable yields. A joint effort with Calgene (Davis, CA) has determined its amino acid sequence by using this enzyme preparation. The sequence shows that B-512FM dextransucrase has 1527 amino acid residues. Once the crystalline form is available, this sequence will be of great importance in solving the three dimensional structure of the enzyme when combined with X-ray diffraction.

By chemical modification with diethyl pyrocarbonate and photo-oxidation in the presence of methylene blue and Rose Bengal, we concluded that there are two essential histidine residues in the active site of the enzyme. This study was consistent with the mechanism of dextran synthesis and the acceptor reactions of dextransucrase proposed by Robyt *et al*[11,13]. In addition we now propose that two histidine residues are involved by donating one proton to each of the two leaving fructose molecules during the formation of

glucosyl-enzyme intermediates; one of these deprotonated imidazole group then abstracts a proton from one of the C-6 hydroxyl groups of the covalently linked glucosyl residue facilating the formation of an $\alpha(1\rightarrow6)$ linkage. The work described in the last two sections of the dissertation on the acceptor reactions has carried the study of the acceptor-site further, by showing the role of each hydroxyl group plays in the binding to the acceptor-site. It seems that the C-2 hydroxyl is the least important in terms of acceptor reaction and the C-4 hydroxyl is the most important since it determines the binding orientation of the pyranoside ring. It is also shown that both the reducing end and the non-reducing end of maltodextrins can accept D-glucopyranosyl residues from the enzyme to form acceptor products. In this work, the idea of the existence of a separate acceptor binding site was developed. However, the exact relationship between the sucrose binding-site and acceptor binding-site is not yet fully understood.

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